

Technical budgets are not elastic

The British government's ambition to reduce the budget of the European Space Agency by a quarter in the next five years is misconceived, commendable though the search for economy may be.

THE British are notoriously and waywardly in two minds about the European enterprise of "ever closer union", but even British enthusiasts seem to have marked out for themselves a role as Europe's bookkeepers. Last year, Britain's Office of Science and Technology (OST) made a fuss about the proposed cost of building the Large Hadron Collider at CERN at Geneva, and won for all member governments membership subscriptions lower than they would otherwise have been. Now OST is on the warpath again, this time seeking a reduction of subscriptions to the European Space Agency (see page 441). The incentive is domestic; its own budget is under pressure. Success could bring rewards to all, and without them being labelled as trouble-makers as the British seem destined, yet again, to be. But the latest campaign could also cause more trouble than it is worth.

The British goal is arithmetically simple; a reduction in real terms of 25 per cent in membership subscriptions within five years. It is easily understandable that the government should have reached this conclusion; the contribution to ESA is more than two-thirds of everything Britain spends on civil space. Free money is so scarce that British scientists cannot always participate in projects they have helped to design. Yet OST seems not to want to cut into ESA's programme, or at least those parts of it to which Britain has declined to contribute. (ESA operates on parallel twin budgets, with an "à la carte" element from which members may opt out.) The weakness in its case is that there is no objective evidence that ESA's operations are 25 per cent more expensive than they need to be. Such evidence as there is allows room for economy, but by a smaller margin.

Even if OST's position is simply tactical, a prelude to a compromise at a later stage, it is unwise. It is not merely that Britain has won itself a rotten reputation for making trouble on European stages, so that protesting at yet another budget will reinforce an impression that wins enemies, not friends. There are also genuine difficulties in dealing with technical agencies demanding that they should carry out specific programmes within budgets reduced by arbitrary amounts; the situation is familiar to applicants for research grants who are told that their applications have succeeded, but that only 75 per cent of the funds can be granted. Often there is no such thing as 75 per cent of a laboratory assistant, or of an electron microscope. Much the same may be true of ESA; a small cut might be an economy, a larger one could damage the programme seriously.

Discovering empirically how much of ESA's budget can

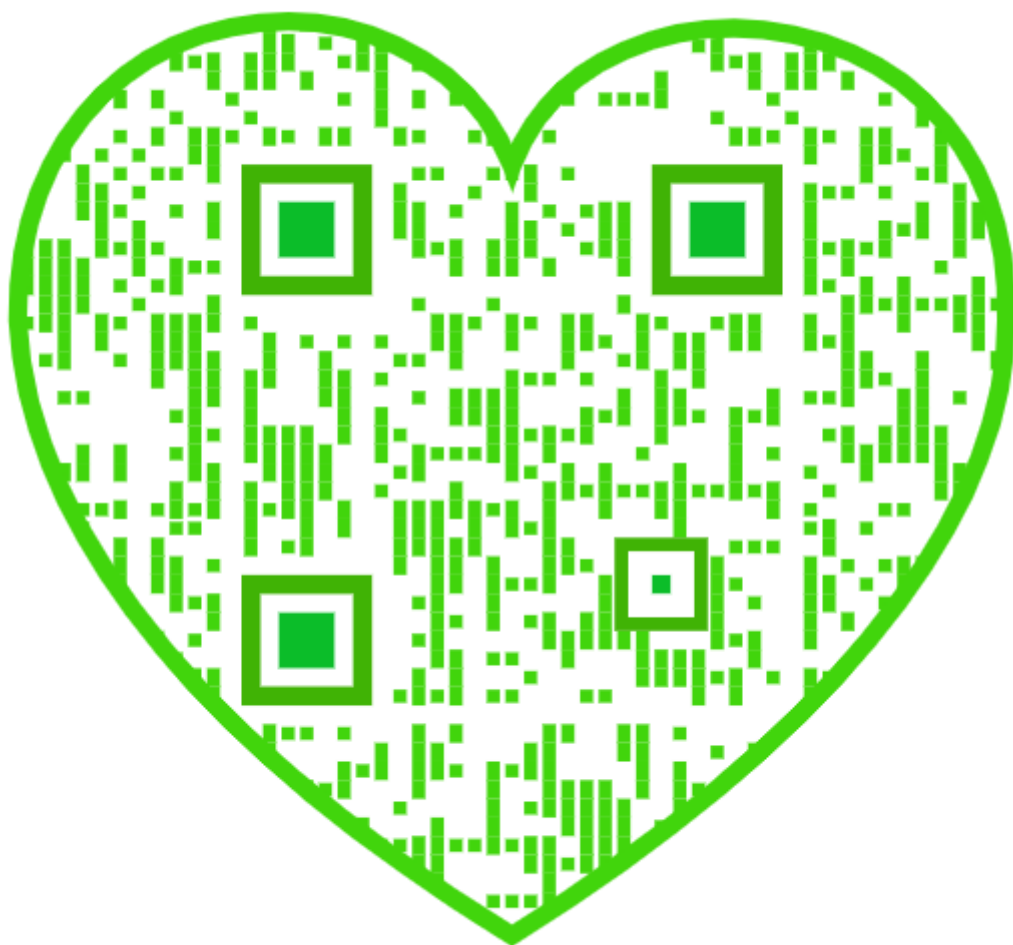
be safely cut is both dangerous and irrational. If Britain indeed has ambitions to be Europe's general-purpose auditor, it should follow those who analyse expenditure carefully, compare the data uncovered with those arising from other sources and should then isolate, for the benefit of ESA's managers and its member-states, where economies might constructively be made. In ESA's case, it is likely that in-house salaries may be greater than those paid to the agency's commercial contractors, which is a case for a policy decision by the agency's council on employment policy. Moreover, audit investigations of this kind need to be conducted on a continuing basis, especially in international organizations where the costs of compromise decisions cannot easily be foretold for decades ahead. But Britain's OST is demanding a once-and-for-all deal whose long-term consequences may themselves be financial extravagances. □

Catastrophe on Sakhalin

Russia still expects too much stoicism of its large population and must learn to look after its people better.

THE dreadful loss of life in last week's earthquake on Sakhalin Island says three things about the condition of modern Russia. First, the dwellings constructed in Soviet times to house those tempted to work in this distant oil field were replicas of the apartment blocks run up in recent decades in and about most Russian cities. Jerry-built, they were inappropriate to their wilderness environment and virtual death-traps in a seismic region such as this. When Russia can generate the resources necessary, it will have to replace much of the housing-stock in which people now live.

Second, vast Russia is poorly equipped to counter the effects of natural disasters. And that is not simply because resources are scarce; the problem is also psychological, born of the deep-rooted conviction that natural and other catastrophes will eventually be got rid of by research and its application, and that rescue is an inelegant stop-gap solution. The third lesson of Sakhalin is linked with the second; a high degree of fatalism is expected of the Russian people, now as it was in Soviet times (see also page 437). In Moscow, which is eight time-zones away, prominent politicians are occupying themselves again with the dignities that go with being a great power. Should they not pay equal attention to the comfort and safety of their people? □



Science in China to face its biggest upheaval since mid-1970s reforms

London. In a move which reflects concerns in the West over linking science to wealth creation, the Chinese government has told scientists in a 700-page white paper that most of the country's research laboratories are to be turned into business units aimed at fuelling economic growth.

The document, whose publication coincides with the sixth anniversary of the Tiananmen Square massacre, also announces plans to spend 80 per cent of China's research budget on "agriculture and industry", and the diversion of 100,000 scientists to "scientific and technological work geared to economic construction".

But government officials claim that they do not intend to neglect basic research. Song Jiang, minister of the state science and technology commission, promised last week that spending on basic research would double to 10 per cent of China's total research budget by the year 2000.

The white paper, said to be the most radical overhaul of Chinese science for almost two decades, carries the stamp of the Chinese president, Jiang Zemin, and other senior members of the Communist party. It confirms earlier announcements that China's science budget will treble from 0.5 per cent of gross domestic product to 1.5 per cent by the year 2000. (see *Nature* 375, 175; 1995). The paper's overall proposals are expected to be put into place next year at the beginning of China's twelfth national five-year plan.

Addressing a specially convened five-day national conference on science and technology in Beijing last week, China's vice-premier, Li Peng, said that, from next year, basic research is to be concentrated among "a group of elite scientists". The rest of the country's researchers, he said, "should merge themselves with businesses", or turn themselves into "financially and developmentally independent businesses providing scientific and technological services".

In his opening remarks, Jiang Zemin said the changes were needed to introduce competitiveness in science "in accordance with

the ideals of a socialist-market economy". He added that "scientific and technological work should always make economic construction its main theatre of operations".

The emphasis on applied science, according to the Chinese president, has already "made outstanding contributions to promot-

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Peng (left): concentrating China's basic research among 'a group of elite scientists'; others should look to business, he says.

Gamma

ing economic and social development, enhancing overall national strength and improving the people's living standards".

The white paper also coincides with a report from the Australian National Academy predicting that China will be the world's second-largest economy and among the leading six trading nations by the year 2005. China has recorded double-digit growth for six years in succession since 1989.

Reactions to the white paper, which effectively amounts to a reversal of a 1978 white paper that sought to strengthen basic research, have been mixed. Not surprisingly, it lacks the wholehearted endorsement of the 90,000-member Chinese Academy of Sciences. The 120-institute academy — the oldest and largest centre of research in the country — has watched its funding decline over the past decade, and will effectively be broken up if the proposals go ahead. Its

members are said to be unhappy at the document's apparent emphasis on technology at the expense of basic research.

In a rare public expression of dissent, Zhou Guangzhao, the academy's president, has warned China's leaders in an article in the Communist party newspaper, *Renmin Ribao*, that economic growth cannot be achieved without investing in basic research. Cutting back on it, he said, would be a waste of rare scientific talent, and is already contributing to the brain drain.

Sean Spingler/Panos Pictures

Others doubt how far the white paper's proposals will be put into practice. Fang Lizhi, for example, the physicist who was a key figure in drafting the 1978 white paper and later become involved in protests at government actions, is deeply suspicious.

Fang, who now teaches particle physics at the University of Arizona, believes the drive to turn laboratories into autonomous enterprises is motivated by a desire to dismember, and thus silence, the once powerful academy, a continuous source of criticism of government policy.

He points out that more than 20 of the academy's most senior members, including Xu Liangying, a prominent historian of science, and Wang Ganchang, father of China's nuclear programme, wrote an open letter to the government last month calling for greater democracy and human rights (see *Nature* 375, 269; 1995).

He also adds that, unlike the 1978 document, the new white paper is thin on detail. "There's far too much rhetoric and ideology," he says, claiming that the white paper is "obsessed" with applied research. "Back in 1978, we had the right balance between pure and applied science," says Fang. "Now, they're damaging a lot of institutions by turning them into businesses."

But Peter Suttmeier, director of the Centre for Asia and Pacific Studies at the University of Oregon, warns against dismissing the document as "just another piece of rhetoric". The white paper, he says, is much too significant to be dismissed as a conspiracy against the academy, which has recently had its budget cut substantially, and has to compete for funds with the newly-established National Science Foundation.

"A lot of institutions that were at the heart of academic life in China are now at risk because of reform policies," says Suttmeier. "The academy is the sort of institution that had a certain logic in a Soviet-style economy. But it has no relevance to the marketplace, and the Chinese face a dilemma over what to do with it." **Ehsan Masood**

Yeltsin promises to boost research budget

Moscow. Russian president Boris Yeltsin, speaking at the first meeting of the newly-created Council for Science and Technology Policy, has promised to increase funding for science to three per cent of total government expenditure in 1996.

In his speech, Yeltsin said that it was important that the funds available for science were increased. But he also said that greater efforts should be made to

attract funding for private sources and to develop scientific discoveries into commercially exploitable products.

Yeltsin also repeated his call for a coherent science policy that would include an acknowledgement of the 'strategic aspects' of scientific and technological development, including the need to ensure adequate supplies of trained scientists, technicians and engineers. □

NASA seeks a better, faster distribution of space data

Washington. The US National Aeronautics and Space Administration (NASA), faced with political pressure to increase its links with the wider community, is urging scientists who fly experiments on its spacecraft to share their data with the public and other researchers more quickly than in the past.

The space agency is also considering privatizing — and perhaps breaking up — its vast archive of data from past space missions. Many scientists say that they support such an idea, provided the service is not scaled back to save money.

Daniel Goldin, the head of NASA, has told space scientists that he thinks the practice of allowing individual researchers exclusive access to taxpayer-funded data should be curtailed or stopped.

Outside scientists working on NASA-sponsored missions, as well as researchers granted time on NASA instruments such as the Hubble Space Telescope, traditionally have a proprietary period during which only they can use the data. This period is typically between six and 18 months, after which the data are archived and made available to the entire research community.

But the recent trend on projects such as the Pentagon-funded Clementine Moon-mapping mission, or the observing campaign for last year's comet impact with Jupiter, has been to release the data immediately. Keen to 'reinvent' its culture, this has now become NASA's preferred model; for example, the agency has asked science teams for the 1997 Cassini Saturn mission to reduce the proprietary data period to as little as nine months.

Stamatios Krimigis of the Johns Hopkins University's Applied Physics Laboratory, a principal investigator on the Cassini mission, supports the space agency's push toward quicker dissemination, saying that in the past some researchers have "sat on their data" for far too long.

Another Cassini team member, Jonathan Lunine of the University of Arizona, agrees that "we should err in the direction of making [the data] available as soon as possible". But, he warns, NASA should also give team scientists enough money and resources to sort out bugs in the data and to format the data for general distribution. Otherwise the released data could be of poor quality.

Defenders of a proprietary period argue that external scientists who spend years developing a flight experiment should have first crack at publishing the results. If the period is too short, they say, team members can spend a considerable amount of time preparing the data for everyone else, and thus lose their competitive advantage.

A new NASA 'science policy guide' released this week, which covers a wide

range of issues affecting the NASA science community, acknowledges that the question of data rights involves "sometimes contradictory factors", from the question of intellectual property rights to the risk of publishing inaccurate results.

The guide, which was written by France Cordova, NASA's chief scientist, and the agency's science administrators, says that data policy for each NASA research project will in future be established on a "case-by-case basis".

Meanwhile, the space agency is looking for ways to hand over the management of its archive of data from past spacecraft missions to a non-government entity — or entities. Much of the data is now kept at the National Space Science Data Center (NSSDC) at the Goddard Space Flight Center in Maryland, which provides tens of thousands of researchers each year with free data over the Internet, or with tapes and CD-ROMs sold at minimal cost.

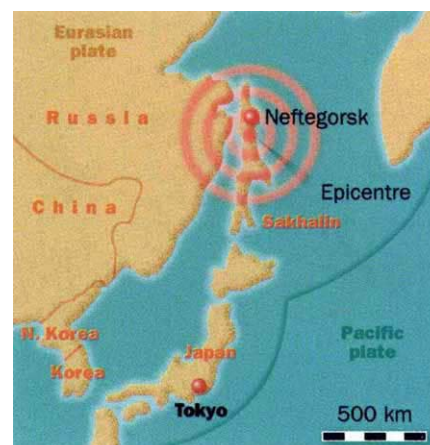
Last week, NASA called for ideas on how the NSSDC could be privatized. Few believe a privately run service would cost the government less; as there is no real "market" beyond the research community, true commercialization is probably not an option. But a private operator may offer a better and more efficient service than the NSSDC. Many scientists say the current system, although it has improved over recent years, is outmoded and difficult to use.

The data archive might be broken up by discipline, with one centre handling high-energy astrophysics data, for example, and another handling planetary geology data. The Space Telescope Science Institute in Baltimore, Maryland, which archives data from the Hubble telescope, could be a model for these kinds of distributed centres.

The advantage of this approach, says Krimigis, is that the data would be treated with "understanding and tender loving care" by researchers who work with them every day, and who can best format them for the rest of the science community.

However these data issues are settled, the new NASA science policy guide — which is due to be made available on the Internet at <http://www.nasa.gov>, under "Hot Topics" — indicates that NASA-funded scientists are no longer expected to serve just the science community, but also the national economy, politicians, and school children, among others.

Under a section called the "Social Contract", the document states that "both the public and the political system expect benefits broader than purely scientific ones to be derived from NASA research programmes and missions". **Tony Reichhardt**



Rough encounter: the Sakhalin earthquake occurred in region where plates meet

Limited knowledge in Russian quake zone

London. Poorly designed buildings, relatively few seismic stations and an earlier reluctance to acknowledge eastern Russia as an active earthquake zone each appear to have contributed to the 2,000 fatalities in last month's magnitude 7.1 earthquake on the island of Sakhalin.

Russia's *Interfax* news agency reported last week that the local prosecutor's office has instituted criminal proceedings for infringing building safety codes after 19 five-storey buildings, constructed during the 1960s in the city of Neftegorsk collapsed.

Russian seismologists, however, are divided over why conventional structures had been built in an area well known for its seismicity (Sakhalin has experienced dozens of earthquakes since records began, including a magnitude 7.1 earthquake in 1971).

Some claim the Soviet authorities leant on geologists to underplay the earthquake risk in the region — despite the fact that such phenomena occur regularly — to save money on otherwise expensive buildings.

But others disagree, claiming the problem was mere ignorance. "We really did not know much about this area during the Khrushchev building era of the 1960s," says one former Russian seismologist now working in the West. "The north of Sakhalin was relatively inaccessible, and our instruments were very inaccurate," he says, adding that surveys carried out at ten-year intervals gradually changed the Russian estimation of the zone's seismicity. "But for a long time seismic zoning for the whole of Siberia was very primitive."

Meanwhile, 60 of Russia's 180 seismic stations — including one at Okha, the town nearest Neftegorsk — have been closed as a result of budget cuts over the past year, according to Oleg Starovoyt, director of the Russian Academy of Sciences' Geophysical Service. Many were in dangerous seismic regions, he says, adding that stations still operating are badly underfinanced. **E.M.**

NIH panel to monitor peer review in action

Washington. The US National Institutes of Health (NIH) are to set up a panel of university scientists and NIH officials to monitor the way in which peer review is applied to NIH grant applications.

The new panel will develop peer review policy for all parts of NIH, and will coordinate the activities of the Division of Research Grants (DRG) — which is responsible for the evaluation of about 80 per cent of NIH grant applications — with peer review administered directly by the various NIH institutes and centres.

It will also periodically scrutinize the various DRG study sections to ensure they are operating fairly and efficiently and are keeping up with new developments in research.

"The panel will be a place where all issues relating to peer review at NIH can be discussed, and people with broad concerns about peer review can be heard," says Wendy Baldwin, deputy director for extramural research at NIH, and chair of the new oversight group. "It is not intended to micromanage the DRG, or to examine individual complaints of abuses in the peer review process."

The formation of the oversight body had been strongly recommended by a group of scientists from both inside and outside NIH to set up to review the administrative structure of the DRG. The committee was chaired by Marvin Cassman, director of the National Institute of General Medical Sciences (NIGMS), and began its work in January. It reported its conclusions to Harold Varmus, the director of NIH, in mid-May, and Varmus announced his decision to create the oversight panel last week.

Cassman says that his committee found no major fault with the internal organization of DRG, which has been an independent division reporting directly to the NIH director since 1946. But it did find several problems that an oversight panel could address, including a feeling that evaluation standards are not consistent across the DRG's study sections and institute review groups. "We felt that an oversight board would help to unify the peer review process for all of NIH, and allow much greater input from the extramural community than we currently have," says Cassman.

His committee also considered whether DRG should be placed under the authority of the deputy director for extramural research. But it failed to reach agreement. Although that would give authority over the largest body evaluating extramural research

to the individual directly responsible for the extramural research programme, some committee members feared it might also be seen as a demotion for the division.

This itself could complicate recruitment of a new DRG director to replace Jerome Greene, who stepped down from this post at the end of March. "In the end we decided to pass the buck [to Varmus]," says Cassman. Varmus says that, for the present, DRG will continue to answer directly to him.

Baldwin is now soliciting nominations for membership of the oversight panel, which is planned to be in operation within three months. Current plans call for 17 members, she says, the majority of whom will be extramural researchers.

US cardiologist found guilty of theft

Boston. Bernardo Nadal-Ginard, a world-renowned cardiologist who formerly headed the cardiology department at Children's Hospital in Boston, has been found guilty on 12 counts of larceny for having stolen more than \$117,000 from the hospital and other organizations.

The verdict was reached after a three-week trial and some 25 hours of deliberation by the jury. Last Monday (5 June), Nadal-Ginard was given a one-year jail sentence, with an additional term in state prison of three-and-a-half to five-and-a-half years, suspended for five years, during which he will be required to carry out 40 hours a week of community service. In addition, the state medical board may revoke his licence to practise medicine.

Scott Harshbarger, the Massachusetts attorney general whose office prosecuted the case, described it as "a classic case of a person exploiting his position of trust for his own personal financial gain". He added: "White collar crime in the health care system costs us all; that's why it is critical we take a very hard line against it."

Harshbarger's office has still to decide whether to seek a retrial on 10 additional counts of larceny on which the jury was unable to reach a consensus. In all, the physician was accused of diverting \$380,000 for personal use (see *Nature* 367, 401; 1994).

According to some reports, the jury became deadlocked on these charges partly because of claims by Nadal-Ginard and his attorneys that the embezzlement stemmed from a psychiatric condition, "Bipolar 2 mood disorder", from which he is said for have suffered for the past 30 years.

They maintained that severe mood swings made him unable to realize that he was committing a crime by taking money from the hospital and two nonprofit foundations, the Boston Children's Heart

In a separate initiative designed to keep abreast of the problems confronting biomedical researchers, Varmus has appointed a 14-member advisory committee to examine the threats to US clinical research. This panel will examine topics such as the growing difficulties in recruiting patients to clinical trials and in financing clinical research as more people join health maintenance organizations instead of traditional fee-for-service insurance plans.

The panel will also look at the changing role of academic clinical research centres, and the training of new clinical researchers. It will be chaired by David Nathan of Harvard Medical School and will hold its first meeting next month.

Robert Taylor

Foundation Inc. and the Cardiovascular Surgical Foundation.

This argument was not used, however, to defend Nadal-Ginard on the 12 charges on which he was convicted. The jury found him guilty of stealing money through a number of schemes from August 1991 to December 1992.

In one scheme, Nadal-Ginard deposited in his personal bank account five cheques made out to the cardiovascular foundation that were supposed to support research and laboratory equipment for children with heart disease.

In another, he used money from the Children's Heart Foundation to pay the salary of the head of a new biotechnology company in which he had invested. The jury also decided that Nadal-Ginard had used Heart Foundation money to make a personal contribution to a Boston museum.

In a civil trial which concluded last December, a US District Court judge ordered Nadal-Ginard to pay \$6.5 million to the Boston Children's Heart Foundation for withdrawing excessive compensation from a pension fund. That decision is under appeal.

Nadal-Ginard, meanwhile, is still technically on "medical leave" from his faculty post at Harvard Medical School. "We have full confidence in the integrity of the judicial process," said Harvard University spokesperson, Joe Wrinn. "The jury considered the evidence and reached its decisions which we respect." The university now plans to review Nadal-Ginard's status at the medical school.

Harshbarger says that his office will "vigorously prosecute" such cases as a way of demonstrating that the law applies "equally to everyone, regardless of someone's socio-economic status".

Steve Nadis

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Varmus: setting up
oversight body.

Mikkel Aaland

Brussels bids for a coordinating role on technology policy

Paris. The European Commission last week unveiled plans for a series of high-technology research programmes aimed at increasing the focus of European Union (EU) research on industrial and social goals.

In particular, Edith Cresson, the research commissioner, and her industry and transport counterparts, Martin Bangemann and Neil Kinnock, announced the creation of joint 'task forces' that will identify and coordinate various public and private research efforts within the member states, the EU's joint Framework research programmes and other European programmes.

The first six task forces will analyse the state of European research in educational software, vaccines and viral diseases, transport, and new generations of cars, trains and aircraft. They will then recommend research priorities based on "intensive consultation" with industry. Other task forces, for example in biotechnology, may also be created.

Although the task forces have also been asked to recommend ways to combine elements of the specific programmes within the fourth five-year Framework programme, which runs until 1999, their main impact will be to help shape priorities within the fifth Framework programme, according to commission officials.

One probable change, they say, is that, in addition to scientific quality, industrial and socioeconomic criteria will be explicitly taken into account when approving Framework projects, resulting in a "double selection procedure". Cresson says that the commission "has made *rapprochement* between research and industry a priority".

She adds that the task forces may eventually propose joint research programmes involving some, but not all member states, within the Framework programme, and recommend that the commission should take part in programmes run by consortia of member states outside Framework, using legal structures similar to that used to run the Airbus consortium.

But such plans are unlikely to materialize in the short term, according to one commission official, who points out that moves towards such 'variable geometry' were rejected as "premature" by the council of ministers in March. Indeed, officials have admitted that the creation of such programmes is likely to face serious "technical, political and management" problems.

The commission's plan to fund research likely to lead to commercial applications is also likely to reopen the heated debate over whether the EU should use taxpayers' money to subsidize industry, given its poor record in the past of picking winners in technology.

Declan Butler

'Science and reason' forum finds enemies all around

New York. The work of scientists is being systematically and effectively undermined by "irrationalist" critics on both extremes of the political spectrum, according to speakers at a forum held in New York last week on "the flight from science and reason".

But the forum, organized by the New York Academy of Sciences and held at the New York Academy of Medicine in Harlem, concentrated chiefly on perceived attacks from the left. Daniel Kleppner, a physicist at the Massachusetts Institute of Technology, said the most serious threat was coming from "the whole post-modernist 'shtick'".

The ire of Kleppner and others has been drawn primarily by social constructivism, the

practicing a few years ago, had decided "not to get into a fight with [Senator Tom] Harkin over alternative medicine". Harkin — a key supporter of biomedical research — has been the most prominent supporter of the Office of Alternative Medicine.

Gross and others attacked that office, which they portrayed as an effort to exempt treatments such as acupuncture and homeopathy from the full rigours of medical science. Gerald Weissmann, of the New York University Medical Center, went further, saying that "fascists and autocrats are trying to overthrow medicine".

Gerald Holton, a Harvard physicist, said that a 1992 draft of the school science teaching standards had explicitly rejected the old, "logical positivist" model of science in favour of a constructivist one. A 1994 version omitted reference to the model, he said, but was still "peppered with social constructivist views".

By the standards contained in the new version, Holton said, "Madame Curie didn't discover radium, she constructed it". The document portrayed advances in science not in absolute terms, but as "changes in commitment of the group". Faced with such challenges, Holton argued that science must develop a better "sense of self", and act more forcibly to defend itself.

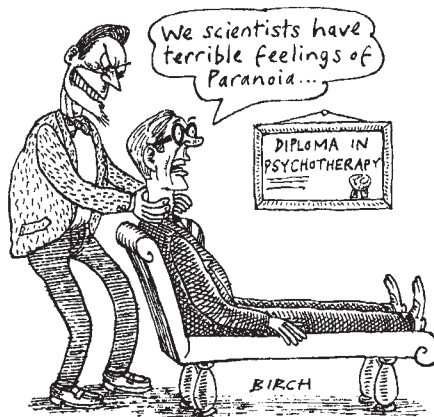
Critics of modern medicine and science — sparsely represented on the platform during the three-day event — fought back from the auditorium floor. Daniel Miller, a Brooklyn psychologist who claims some success in treating cancer patients, said the meeting was "a power structure sitting in a pretty building trying to defend itself". David Guston, a specialist in science and public policy from Rutgers, complained that speakers' characterizations of critics in the humanities had been "totally overblown".

One speaker who appeared to agree was Langdon Gilkey, a theologian at Georgetown University in Washington. "I feel the lack of a self-critical faculty here," said Gilkey. "The genuine criticisms of science have been rendered into the 'cookie' class, as if they weren't real."

He warned a science community which, he said, was more omnipotent in modern culture than it realized: "Powerful communities, unless they criticize themselves, will face mounting criticism from outside."

Dudley Hershbach, the Harvard chemist and one of three Nobel prizewinners recently 'deconstructed' in three critical television documentaries, called on scientists to show some self-effacement in dealing with criticism. Science, he said, should be "a partner of the humanities in the search for wisdom of all sorts".

Colin Macilwain



intellectual school that seeks to describe science as a collection of ideas constructed by one social group — namely natural scientists — and of no greater intrinsic value than any other such collection of ideas.

According to Norman Levitt, a mathematics professor at Rutgers University, New Jersey, who co-organized the conference, constructivists think "science is no better in its justifications than other disciplines — so it is a species of fraud *ipso facto*".

Speakers acknowledged that few working scientists are even aware of this assault on their worth. Nevertheless, they claimed that its pernicious effects are spreading quickly through universities and (more recently) schools, where they will eventually undermine the standing of science and medicine.

Two recent developments were seen as symptomatic of a wider malaise: the establishment of an Office of Alternative Medicine at the National Institutes of Health (see *Nature* 370, 591; 1994), and the allegedly constructivist leanings of draft standards for science teaching in US schools.

Paul Gross of the Centre for Advanced Studies at the University of Virginia, who organized the meeting with Levitt, said that the medical establishment, discouraged by its failure to restrict the work of chiro-

Whaling nations come under fire over 'scientific' practices

London. Norway and Japan have come under pressure from the International Whaling Commission (IWC) to stop what many consider to be commercial whaling dressed up as scientific research.

At a meeting in Dublin, Ireland, last week, the members of the IWC passed a resolution calling for all research inside whale sanctuaries — including the controversial Southern Island Sanctuary — to be non-lethal.

This follows growing evidence, based on DNA analysis, that meat taken from whales killed in these sanctuaries for 'scientific' purposes is finding its way on to the open market, particularly in Japan.

The meeting also passed resolutions aimed at curtailing scientific whaling in general. These included the demand that such research should be non-lethal, except in exceptional circumstances, and that governments should either refuse or revoke permits for research 'culling' that fails to meet such criteria.

Under the rules of the IWC, permits can be issued allowing a limited number of whales to be killed for 'scientific research'. But many whaling critics feel that this is providing a loophole for the outlawed trade in whalemeat.

The minke whale population was declared 'protected stock' in 1985 when the IWC felt that the species was in danger of extinction because of excessive whaling. Norway, however, is still allowed by the IWC to kill a certain number of whales for 'scientific' purposes.

Thirteen countries — including the United States, Australia, New Zealand and most European member states — drafted a resolution which was passed by 21 votes to six calling on the Norwegian government to halt all whaling activities under its jurisdiction, and to reconsider its objection to the moratorium on commercial whaling.

Japan, like Norway, also reserves the right to import meat from whales killed for scientific research. But PCR tests on market-seized samples have apparently uncovered a pattern of widespread illegal whalemeat being traded in the north Pacific Ocean.

Following reports from its scientific committee, the IWC meeting passed a resolution requiring member states to introduce random DNA and isozyme testing, and to monitor, declare, register and dispose of whalemeat stockpiles. Despite its wide-ranging objections to IWC policy, Japan cannot afford to give up its membership, as that would mean losing valuable fishing rights in US waters.

Sara Abdulla

Materials research unit to break the mould in Japan

Tokyo. Ten Japanese companies have joined forces with two research laboratories, in Japan and the United States, to set up a profit-making contract materials research centre. The International Center for Materials Research will aim to identify and solve industrial materials problems with the help of university researchers.

This unusual example of academic/industrial cooperation in Japan comes at a time when Japanese manufacturers, faced with the need to keep workers at their domestic factories busy while much production is moving elsewhere in the Pacific Rim, appear to have decided that diversification into high technology may yet be their best bet, particularly as cash-strapped companies are reluctant to increase their fixed costs and to employ more researchers.

One of the two laboratories that make up the new centre is located at the centre's headquarters at Kanagawa Science Park, Kawasaki, Japan's first venture business 'incubator', and already employs five PhD-level scientists. The other laboratory will be based in Lexington, Kentucky, and will begin operations with a director and two PhDs next month.

Half of the consortium backing the centre, which will have a paid-up capital of ¥30 million (\$353,000), consists of large, established companies such as the textile-makers Toray and Teijin, on the lookout for new business opportunities.

The other half are small, rapidly-growing companies that are strong in a particular technology but want to apply their expertise in other areas. They include Nitto Denko, whose speciality is sealing resins for microchip packages, and Dekko New Industrial, a microencapsulation specialist.

One of the attractions of the centre to the consortium members is its president, Tadashi Sasaki, who for more than 40 years has been one of Japan's most astute technological visionaries. In the early 1950s, for example, Sasaki was the first Japanese to license the transistor (for Kobe Kogyo, now part of Fujitsu).

As research and development director for the electronic appliance maker Sharp, Sasaki identified key technologies such as the liquid crystal display. Since his retirement from Sharp, he has been an adviser to SoftBank, a software distributor that is one

of Japan's fastest growing companies. Now, at the age of 80, the indefatigable Sasaki — his subordinates call him 'Dr Rocket' — has embarked on yet another venture.

Sasaki explains that the decision to focus on materials research is because materials such as steel and silicon are the platform on which Japan's industrial structure is based. "Marketing, assembly and components have all gone offshore because of the high yen," he says, "if materials go too, then our industrial structure will disappear."

His idea is to develop advanced functional materials that are too difficult for newly industrialized countries such as China to produce. Sasaki reckons it should be possible to manufacture high value-added materials such as conductive polymers in Japan for a few years "until the Chinese can catch up".

The centre will work on two types of development. One will be to develop materials likely to meet future markets; the second — more important — strategy will focus on developing materials specifically for existing market needs.

The centre's ten-person advisory board will be headed by two physicists, Mildred Dresselhaus of the Massachusetts Institute of Technology (MIT) in the United States and Harold Kroto of the University of Sussex, England, both known for their work on carbon 60. Their involvement explains why, in addition to conductive polymers, carbon 60 is the other material chosen by the centre for its initial project.

Peter Eklund, professor of physics at the University of Kentucky, and the director of the centre's new satellite Lexington laboratory, has been working on carbon 60 for the past five years. During the mid-1970s, Eklund was a postdoc in Dresselhaus's group at MIT, where he met and became friends with Nobuyuki Kambe, now senior managing director at the new centre.

Before helping to set up the centre, Kambe spent two and a half years as a strategic planner for new business at Nippon Telegraph & Telephone. "Up to now, research and development has been done within one company," Kambe says. "but that era is over." In future, he believes, small, collaborative outfits like the centre will become popular because of their potential to reduce research costs and shorten development time.

As the first such company in Japan, however, the centre faces novel problems as well as opportunities. One major concern is how to handle intellectual property rights. Patent royalties will be split four ways between the inventors, the centre, participating companies and universities that have helped to develop the technology.

Bob Johnston



Sasaki: visionary now looking to materials.

UK gets rough ride over call for space cuts

London. An independent review of a satellite mission being planned by the European Space Agency (ESA) has concluded that, if different procedures had been adopted by both the agency and the scientific community, the estimated costs of the mission could have been reduced by between 10 and 15 per cent.

But the author of the study says he has found no evidence to support claims by British government officials that the overall costs of ESA's space science programme could be reduced by 25 per cent without a major impact on its activities' outcome.

The study was based on the proposed Satellite Test of the Equivalence Principle (STEP) mission, designed to test the assumed equivalence between the inertial and gravitational mass of bodies. It comes at a time when ESA is under pressure from several member states to find ways of saving money in its \$3.5-billion budget.

Some possible economies — such as a 20 per cent reduction in permanent staff — are said to have been outlined in two recent audits of the agency, one by the management consultants Andersen Consulting, the other by Price Waterhouse, which were delivered last month.

ESA science missions are generally accepted to be well managed and run in a relatively cost-conscious way. But even agency officials acknowledge that ways of reducing costs might be found, for example by increasing the accuracy of studies at an early stage in the planning of missions.

Their efforts have been endorsed by ESA's principal advisory panel, its science programme committee (SPC). Last week, the members of the committee agreed unanimously on the need for enhanced efficiency in the agency, given increased demands from the scientific community and budgetary pressures in various member states.

David Southwood, professor of physics at Imperial College, London, and chairman of the committee, says that a "large majority" of its members, meeting in Paris, favoured consolidating such efficiency gains into the scientific programme.

The main exception is Britain, which is seeking a 25 per cent cut in its subscription to ESA over the next five years, arguing that its current subscription now dominates its space budget, thus undermining its national and binational space activities.

Defending Britain's position, Sir John Cadogan, the director general of research councils, claims it was largely UK pressure that led to a reduction of 1 billion Swiss francs (US\$860 million) in the projected costs of building the Large Hadron Collider at the European Laboratory for Particle Physics.

Officials from the UK Particle Physics and Astronomy Research Council are now pointing to the study of the STEP mission,

carried out by Mike Cruise of the University of Sheffield at the invitation of ESA, as evidence that cuts of a comparable magnitude are feasible in the space agency.

In particular, much is being made of the difference between the figure of 426 million accounting units — roughly \$550 million — that has been used as an initial estimate by ESA of the costs of the mission, and the 294 m.a.u. that Cruise claims it could cost under different circumstances.

But Cruise himself acknowledges that some of his assumptions — for example, that salary costs would be the 'mean' of those in national space laboratories, not for ESA staff — are unrealistic under present ESA rules, while the space agency's figure has a built-in margin of error.

A fairer comparison, he suggests, is between his figure, perhaps adjusted slightly upwards, and one closer to the 345 million accounting units that ESA officials have been set as the target cost of STEP, a net difference of 10 to 15 per cent. "My study has shown that there are modest savings available within the ESA system, but I have found no justification that these could be as high as 25 per cent," says Cruise.

Coincidentally — but perhaps significantly — the reduction Cruise says would be feasible is close to that being proposed by Germany for ESA's space science programmes. This would be the result of adopting Germany's suggestion that the budget be

kept level in cash terms for the next five years — that is, would shrink in real terms by the amount of inflation.

Germany, like Britain, is facing pressures on its domestic space budget as a result of economic difficulties. But it is also facing the need to fulfil its commitment to play a significant role in the international space station, to which any savings in the space science budget might be directed.

In contrast, France and some other ESA member states — several of whom pay their subscriptions out of their budgets for industry rather than for research — are insisting that any savings produced through increased efficiency should be fed back into the space science programmes themselves.

Britain's position is very different. Indeed, PPARC has given no commitment that savings in Britain's contribution to ESA's science programmes would be reallocated to the domestic space programme, leading to fears that the money would be used for other research sponsored by the research council, for example in particle physics of ground-based astronomy.

The fact that Germany's proposals go half-way towards Britain's demands indicates that room exists for compromise. But the gap is still substantial, and bridging it will require a political accommodation that is unlikely to materialize before Europe's space ministers meet in October.

David Dickson

US and India sign ocean data deal

New Delhi. The Indian government, having refused for almost eight years to provide the United States with 'real-time' weather data gathered by its INSAT satellites over the Indian Ocean, last week agreed to do so — but only under certain conditions.

India has three communications satellites in geostationary orbits, each equipped to collect cloud and weather data. The United States has, since 1986, been asking for half-hourly data which, it says, are essential for climate-modelling and weather forecasting. But after objections from the Indian military authorities, the Indian Meteorological Department has been supplying only low-resolution, one-day-old data recorded on magnetic tapes.

Officials had argued that high-resolution data could be used to extract information on ship and aircraft movements in the Indian Ocean. Some government scientists are also known to believe that meteorological data about the Indian monsoon are too sensitive to be shared with other countries because of their potential commercial implications.

But last week James Dodge, head of the Mission to Planet Earth office at the

National Aeronautics and Space Administration (NASA), and the chairman of the Indian Space Commission, K. Kasturirangan, signed a 'statement of intent' to exchange INSAT data with that from US weather satellites.

According to government officials, India has now agreed to provide real-time INSAT data on a 'need-to-know' basis. US scientists will have to propose specific projects that justify the need for such data, which will be released only after projects have been approved by the Indian side.

The data will be provided only for the duration of specific projects, and will be transferred through a high-speed telecommunications link. India has insisted that NASA should not disseminate the data, and use them only for specific purposes.

In exchange, the United States has offered to share data from its Tropical Rainfall Measurement Mission satellites, which will provide rainfall measurements over the entire tropics, and help India enhance its monsoon forecasting. India will also gain access to archival climate and weather data maintained by NASA since 1978.

K.S. Jayaraman

'Euro-lab' scientists seek fairer deal on pay

Munich. Labour relations in Europe's international laboratories are becoming strained as their scientists, who are not allowed to join trade unions and have no collective bargaining rights, are taking their complaints about the way salary claims are being handled to the International Labour Organization (ILO) in Geneva.

Most scientists working in organizations such as the European Southern Observatory (ESO), the European Molecular Biology Laboratory (EMBL) and the European Laboratory for Particle Physics (CERN) are paid considerably more than those working for national research bodies — typical researchers may receive a tax-free salary of almost \$70,000 a year — to compensate for the disruption of living abroad.

In recent years, one way in which governments have tried to keep down the costs of such organizations has been by restricting salary increases. But their researchers are now claiming that such moves conflict with accepted guidelines covering staff working for international bodies.

Later this month, for example, the council of ESO, whose headquarters are in Garching, near Munich, will decide whether to implement the recent ruling of an ILO administrative tribunal that it should raise salaries which, according to the ILO, have been kept unfairly low since 1992. According to its statutes, ESO has agreed to refer any salary dispute that cannot be resolved internally to the ILO.

Staff at the EMBL, who believe that they are in an identical position, are also threatening to take the Heidelberg organization to the same administrative tribunal if they do not receive a compromise pay adjustment in the next weeks.

Both claims come at a difficult financial time for the two bodies, which are trying to expand their research efforts despite

constrained budgets. Some argue that EMBL in particular could face major financial difficulties if the staff claim is met in full. But many scientists complain that the absence of an agreed system for adjusting salaries has left them vulnerable to budgetary constraints.

The problem is relatively new. In the early 1980s, both ESO and EMBL agreed to base their annual salary increments on the index recommended by the Coordinated Organizations (CO). This is a committee within the Organization for Economic Co-operation and Development in Paris which makes recommendations on pay for bodies such as the North Atlantic Treaty Organisation and the European Space Agency.

In 1993, however, the ESO council decided to implement only two-thirds of the CO recommendation, and to make this retroactive to 1992. It pointed out that it is not a member of the CO itself, and that the clause in its constitution declaring its intention to follow the CO recommendations includes the rider that the council will make the final decision on pay matters.

But this defence has been rejected by the ILO administrative tribunal. In a ruling in February on two complaints from ESO staff members, the tribunal argued that ESO had set a precedent on pay procedures over the previous ten years on which staff had come to rely. It concluded that ESO should raise salaries to the level they should have been if the CO recommendations had been followed, and compensate fully for any consequent loss of earnings.

ESO refuses to say how much this will cost. But a spokesman, Richard West, says the council has little alternative to paying up, as its member states have already

agreed to accept ILO's rulings.

The ESO council will now decide how to avoid similar problems in the future. "Any agreement which is fair, transparent and stable is okay with us," says a representative of the staff association, claiming that "money is less important than principles, particularly in situations [like ours] where there is no right to collective bargaining or unionization". He acknowledges that staff are well-paid, but says they want to ensure that ESO sticks to its rules of procedure.

EMBL also abandoned its automatic adherence to CO recommendations a few years ago, and its staff association is now discussing the implications of the ESO ruling with a working group set up by the council of the laboratory.

Full implementation of the ILO's recommended indexation would add DM2 million (US\$1.4 million) to this year's salary bill, says Barton Dodd, head of EMBL's administration. Back pay to mid-1993, when the disparities between recommended and actual pay increases began, he says, would cost EMBL a further DM2.5 million.

But Dodd still believes that salaries must remain competitive with those offered by national laboratories. "We ask people to disrupt their lives by coming here for relatively short periods — no more than nine years — and we have to pay a premium to get them here and keep them here", he says, arguing that if rises remain lower than inflation, EMBL salaries will lose their competitive edge.

Both the EMBL administration and the staff union hope to reach a compromise on pay. But obtaining the necessary approval of EMBL's member states may not be so easy, as they are unlikely to accept a settlement based on the ILO ruling on ESO. The main players — Germany, France and the United Kingdom — are hoping that the ILO would establish a new precedent if EMBL staff brought a similar case.

But whatever the outcome, says a spokesman for the German delegation to EMBL, member states are unlikely to pay out until required to do so, as the money involved — around two per cent of the annual budget — could undermine its ambitious research plans. EMBL council is expected to make a decision at its meeting at the end of this month.

Jean-Christoph Olivo, of EMBL's staff association, says that scientists are aware that the increases they are seeking could cause financial difficulties for the laboratory. But he says that they feel that they are being exploited, as EMBL has reneged on its agreement to follow CO indexation, and that if a compromise cannot be achieved, they feel they have no alternative but to refer their complaints to the ILO.

Alison Abbott

CERN researchers may appeal again

Munich. Scientists at the European Laboratory for Particle Physics (CERN) in Geneva have taken salary claims to the ILO every year since 1992, but so far without success; CERN never agreed to follow the salary recommendations of the Coordinated Organizations (see above), preferring to base its indexation on the inflation rate in Geneva.

When wages were kept below this rate at the request of CERN council in 1992, the staff association appealed to the ILO. But the organization ruled that, technically, CERN had no legal obligation to meet the criteria on pay increases it set itself 15 years ago. "In that respect they differ from the methods that other

organizations follow", concluded an ILO tribunal.

But in the light of the recent ruling against ESO that the precedents which were followed in previous years should be adhered to, the staff association is appealing once again to the ILO, claiming that their expectation of pay increases based on the Geneva inflation rate has been breached.

CERN pay has been frozen for the next few years, as the laboratory seeks to hold down costs in order to pay for the Large Hadron Collider. But the administration has recently launched an internal review of its employment conditions.

A. A.

Panel offers compromise on food labelling

Paris. The group that advises the European Commission on ethical questions related to biotechnology has recommended that food be labelled to indicate when its composition and characteristics have been "substantially modified" by genetic engineering techniques. But it says such labelling is "inappropriate" when changes are insubstantial.

The recommendation conflicts with the position taken by the commission itself in a draft directive on novel foods, which opposes systematic labelling on the grounds that it would "tend to stigmatize biotechnology" while providing little useful information to the consumer. That is also the general position endorsed by the US Food and Drug Administration (FDA) in 1992.

The ethics advisory group, set up to help the commission deal with divisive issues raised by genetic engineering, was itself divided, and the final 'opinion', accepted unanimously by the group's members and published last month, is a compromise.

It endorses current regulatory thinking that the safety of products should be "scientifically assessed and assured". But it also says that, exceptionally, consumer demand for information about biotechnology justifies labels indicating the process used, even though — in an attempt to avoid stigmatizing biotechnology — it argues that such labelling should be restricted to cases involving "substantial change".

The Brussels-based Confederation of the Food and Drink Industries wants novel foods labelled only where a change in composition has substantially altered either a food's nutritional value or the way it is metabolized in the body.

But the ethics group's recommendation has been welcomed by the UK pharmaceutical company Zeneca — formerly part of ICI

— which markets a tomato that has been genetically engineered to delay softening. "It's all about choice", says Nigel Poole, regulatory affairs manager at Zeneca. Telling a consumer that he or she does not need certain information will only raise suspicion, says Poole. "We are going to inform the consumer; we are proud of our tomato."

Zeneca's position reflects a growing trend among those using genetic engineering techniques in food production to introduce labelling in response to consumer demand, rather than regulation. Indeed, Poole, in common with others in the industry, feels that the heated debate over whether such food should be specifically labelled will eventually subside as more such products arrive on supermarket shelves, and consumers increasingly judge them on their nutritional and culinary merits.

For example, although 1,500 US chefs have agreed not to serve genetically engineered food (see *Nature* 359, 8; 1992), Calgene's Flavr Savr tomato is reported to be selling well in the United States, as is a vegetarian cheese produced by the UK Cooperative and Wholesale Society which is based on recombinant chymosin — and voluntarily labelled as such.

This trend appears to be confirmed by the fact that almost half of those interviewed in a survey carried out by the Dutch consumer research institute SWOKA said that they did not need to be informed that a food had been produced by genetic engineering techniques, providing it had been approved by a regulatory authority.

A further quarter said merely that they would like to know when such foods came on to the market. Only one-fifth wanted biotechnology foods to be specifically identified on their label.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Genetically-engineered potatoes: should they carry labels detailing techniques used?

Many in the food industry attribute part of the controversy over genetically engineered foods to the "unfortunate" fact that one of the first products to come to market was milk produced using bovine somatotropin (BST), which offered clear advantages to large dairy farmers — but not to consumers. "We started off down the wrong track with BST," says one official of the European Commission.

At the same time, others argue that the recommendations of the ethics group, which draw on assumptions about the consumers' 'right to know', beg the question of the extent to which governments should give precedence to the opinions of vocal pressure groups over 'scientific soundness' in handling complex technological issues.

Claims for the 'right to know' are both seductive and difficult to counter, as the alternative appears to be self-regulation by an unaccountable élite. But, says one critic, the concept is "misleading and simplistic".

Labelling, he argues, could require the publication of information considered irrelevant by regulatory authorities to genuine concern about safety, by potentially causing anxiety among consumers. By highlighting foods produced by biotechnology, pressure groups can create anxiety over a "non-issue" he argues, and stigmatize such products.

The ethics group has apparently rejected this argument. But its recommendations may be unenforceable. Flour, for example, may be ground from several silos, each holding wheat from several farms.

One commission official adds that, if one process is to require labelling, then logically other processes should be identified on food labels as well. Rather than labelling milk as having being produced using BST, he argues, might it not be more important to mention which herbicide was sprayed on the meadow the week before the cow was milked? How long, he asks, can a label be? **Declan Butler**

Curtain descends on CSIRO dispute

Sydney. A bitter dispute between scientists and senior management at Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO), has been settled — but not before questions had been raised by members of a parliamentary committee whose inquiry last year strongly criticized CSIRO management.

Senator Alan Ferguson, who headed the inquiry, said last week that he had received a very "non-committal" answer from Peter Cook, the Minister for Science, about problems in the CSIRO. But he added that the acting head of CSIRO, Roy Green, had agreed that there would be no repetition of the incident that sparked the dispute.

In that incident, the chiefs of five CSIRO divisions — its major operating unit — had each read a letter to their respective staffs apologizing to the CSIRO

board for using the phrase "conflict of interest" in comments submitted by the staffs on an internal management report (see *Nature* 375, 268; 1995).

Although staff say they were referring to the fact that all board members (apart from Green) also work for other organizations, the board is said to have interpreted the phrase as complaining of financial conflict. But the board's response led to staff accusations that the chiefs had been forced to apologize.

According to Ferguson, Green has now agreed to work directly with staff on the organization's problems, particularly on CSIRO's structure and management, and its internal communications. In return, he and other members of the parliamentary committee have agreed not to reopen last year's inquiry.

Mark Lawson

French journalism defended

SIR — For the past several months, *Nature* has made virulent attacks on the behaviour of French judges, police and the press over the so-called “contaminated blood scandal”. The mistakes and confusions in the articles *Nature* has published disregard the elementary verification process that you recommend to researchers.

But a recent Opinion (*Nature* 373, 546; 1995) insulted us, journalists of the popular press, by comparing us to the *tricoteuses* of the French Revolution, who revelled in watching the victims being executed.

You accuse us of “exciting the executioners”. We are reluctant to believe that the latter term is meant to apply to the judges concerned, whose judgements are based on a precise and well documented file. As for ourselves, we feel that we have fully played our part as a counterweight in a case where medical, political and financial interests intermingle.

Our enquiries have enabled us to shed light on damning and undisputed documents relating to decisions taken between 1983 and 1985 by those in charge of the health system. We chose our words with care and moderation, and our analysis of the errors and faults was always supported by solid arguments. The four people judged in two successive trials were blamed for keeping on the market, in 1985, products they knew were likely to be contaminated with the AIDS virus, while a calculation made by Dr Jean-Pierre Allain at the time showed that at least one extra haemophiliac would thus be infected each day as a result. That calculation is still valid today, as four statistical studies later estimated that between 200 and 300 haemophiliacs were contaminated in 1985. We have evidence showing that those decisions were deliberate*.

We have never contended that only four people were involved in this scandal but have argued that other people with medical and administrative responsibility should also answer for their actions before the Court to enable everyone to understand the mechanisms that led to such a failure of our health system. The preliminary enquiries now under way will probably provide an answer to that question. As far as we know, nobody was dragged to the guillotine, as capital punishment was abolished in France in 1981. Your insulting comparison thus turns out to be based on a major historical mistake. As to the analogy between the work of the Paris judges, accomplished in accordance with the basic rules of human rights, and the arbitrary decisions made by the revolutionary juries, the image is inappropriate.

Strangely enough, you fall into line with several transfusion authorities that have

been clamouring for an international commission, supposedly above ordinary justice — another insult to democracy. We would be interested to see which independent international experts would back the medical decisions made in 1984–85. Finally, both Britain and France have a history of what may be called the *élite* approving the worst revisionist theories.

Gérard Badou (*L'Express*), **Béatrice Bantmann** (*Libération*), **Hélène Cardin** (*France-Inter*), **Anne-Marie Casteret** (*L'Événement du jeudi*), **Guillaume Malaurie** (*L'Événement du jeudi*), **André Mazzolini** (*Libération*), **Hélène Mollière** (*Europe 1*), **Anne-Pierre Noël** (*La Cinq*), **Jean-Luc Nothias** (*Le Figaro*), **Françoise Parinaud** (RTL), **Vincent Olivier** (*Le Parisien*), **Michel de Pracontal** (*Le Nouvel Observateur*), **Bernard Seytre** (journaliste indépendant)

*Letter from J. P. Allain dated 19 January 1985; Memorandum from Dr J. B. Brunet to Professor Roux, head of the DGS (Direction Générale de la Santé: General Health Management), dated 12 March 1985; CNTS internal memoranda in May, June and July 1985; Letter from Dr Garretta to the DGS dated 9 May 1985. Report of the meeting of 29 May 1985 at the CNTS (Centre National de Transfusion Sanguine: National Centre for Blood Transfusion) and so on.

Parents' rights

SIR — John Godfrey has articulated a reasoned concept of the gradual rather than the instantaneous development of the human person. His Commentary (*Nature* 373, 100; 1995), while written from an independent point of view, is in line with the views of many Roman Catholic doctors. It is important that the issue should not be regarded as being irrevocably closed and that the legitimacy of conflicting views should continue to be tolerated.

In Ireland, many parents of genetically determined handicapped children look for antenatal diagnosis, and some, in the light of their own knowledge and experience, opt for termination. Older parents are frequently angry if they have a Down's syndrome baby without having had the option of antenatal diagnosis. These observations are based on a long experience of clinical practice. Children vary in height, weight and intelligence and we are accustomed to recognizing a wide range of acceptable values. In the same way, parents vary in their endurance, their insight and their coping capacity. An elderly and ascetic priest once defined this clearly to me in terms of the need to respect the limitations of parents. Parents' endowment is in the gift of the Almighty and parents have an inherent right to have their God-given limitations acknowledged and should not be expected to perform beyond the limits of their endowment.

As long as it remains an open question whether the starting point of actual as against potential human development is

instantaneous or gradual, parents can still be supported in good faith in whatever difficult decisions they take in relation to abortion. We should not interfere with their right to their limitations.

There has been disquiet among doctors about the consolidation of fixed positions in these matters. In 1983, when a referendum was held in the Republic of Ireland on the amendment of the constitution so as to prohibit legislation on abortion, seven professors of paediatrics publicly expressed their concern. All but one were Roman Catholic.

When parents unburden themselves behind the closed doors of the consulting room they have a right to be heard, and on their own terms. Godfrey restates a basis for one of the options for the ethical decision-making process and so is to be welcomed.

Abortion has been listed as a basis for automatic excommunication in the Roman Catholic church. In the Republic of Ireland the ratio of abortions to deliveries is close to 1:10. On this basis there must already be 30,000 excommunicated women in the Republic. By the year 2000 there will be 50,000. Exclusion of these women from the rites and the formal fellowship of the church turns on the insistence that there is only one answer to the question of the point of inception of human life.

If this issue is not left open, sexually active women of child-bearing age will have to conditionally baptize their menstrual products each month in case the tiny morula of one of the 50–80 per cent of spontaneously aborted two-week-old fetuses should be flushed away unmourned.

O. Conor Ward

University College, Dublin, Ireland

Necessary brain

SIR — Benjamin Libet suggests (*Nature* 375, 100; 1975) that “without a functioning brain there is no human person”, and then equates the status of an embryo with that of “an adult whose brain is dead” and from whom, incidentally, tissues “may legally be removed for transplantation”. Following Libet's argument, one could remove tissues from an embryo (or damage it through discharge of industrial pollutants) without fearing legal action when the embryo grows up — one would never have harmed a “human person” *sensu* Libet.

Peter Forster

I Heinrich-Pette-Institut,
University of Hamburg, Germany

Correspondence

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Virtue in now-antiquated textbooks

General relativity is not everybody's interest, but there is much to be said for rereading Tolman's monograph on the subject, first published in 1935, not just for what it says but for what it fails to say.

OCCASIONALLY, most people find themselves forced to read through books and papers they have not looked at for decades, in a kind of instant refresher course in a forgotten subject. It is always a frustrating experience. The old textbooks have the advantage of being familiar, even to the extent that the meaning of the old diagrams stands out without help from the legends beneath them. On the other hand, the old books that have occupied shelf-space all these years have probably long since been superseded by much more effective ways of learning. It might even be discovered that the old books embody precisely those obscurities that made the subject difficult to understand in the first place — and which have made the refresher course necessary.

What follows is an account of an attempt to learn about general relativity in a hurry, almost exactly 50 years ago. The occasion was a series of seminars given at Oxford by the relativist E.A. Milne, whose claim on public attention was the doctrine of "kinematical relativity", which holds that the puzzling business of Lorentz transformation in special relativity tumbles out from a careful accounting for the times spent by light in travelling to and from distant moving objects. Milne had had a bad time in captivity during the Second World War, and had returned the worse for wear.

His seminars, on Tuesday and Friday afternoons in a room at the end of the first floor of the Clarendon Library, caught the attention of half a dozen people with exceedingly diverse interests. One of the most faithful was M.J.S. Dewar, a theoretical chemist who has spent most of his working life at Austin, Texas. Milne's style was Socratic. He enjoyed his seminars best when there was an argument. That tendency afterwards became apparent in Milne's public exchange of vitriolic correspondence with Herbert Dingle, which entertained a year's cohort of *Nature* readers in the early 1950s.

Of necessity, textbooks on relativity were then few and far between. My college library had only two — Eddington's *Relativity, Protons and Electrons* and Tolman's *Relativity, Thermodynamics and Cosmology*, both published in the mid-1930s. Eddington's book is a startling read; among other things, it calculates that the number of bosons in the Universe is between 10^{49} and 10^{51} . One Friday, Milne was talking about Dirac's ideas on the importance of large numbers (not yet formalized) and it

seemed natural to ask whether Eddington's number might be one of them. Milne's response was to demand an oral summary of Eddington's book the following Tuesday. Sadly, the only book from which a little general relativity might be gleaned that was borrowable over the weekend was Tolman's. The copy that has occupied shelf-space ever since is identical, but part of the 1966 reprinting.

For what it is worth, the book would not have been a bad choice. In the retrospect of a present need for a refresher course (unconnected with what follows), Tolman's interest in the relativistic definition of entropy must have been a great source of confusion all those years ago: it would have been irrelevant to Eddington's argument. But the essence of Einstein's general relativity is all there, if dryly. Beginners would have to work hard to get to the bottom of such things as the notion of the parallel displacement of vectors in curved space-time, for example.

But the real interest of Tolman's book lies in what it says, or rather does not say, about cosmology. First published in 1935, the text was probably evolved just a few years after Hubble's proof (or, rather, announcement, for some dispute the adequacy of the data) in 1930 that most other galaxies are receding from our own, and at a speed proportional to their distance.

More striking still, Tolman does not mention Aleksandr Friedmann, the Russian who in 1923 produced the solutions of Einstein's equations that are now the basis of most cosmological models; they languished in the Russian language until about the publication of Tolman's book, and are now known as the Friedmann-Robertson-Walker solutions after their rediscoverers in the West. But Tolman has almost the next best thing, a discussion of the reasons why the Einstein static solution (with his cosmological constant included) must be thermodynamically unstable and why, for cosmologists, the choice must be between a continually expanding Universe and one that first expands and then contracts.

It is curious that the 1930s were almost dog-days for general relativity. It had been different a decade earlier. The appearance of Einstein's general relativity in 1915 stimulated a spate of interest. Minkowski produced a calculation of the gravitational field around a massive object, de Sitter a model for a Universe devoid of matter whose space-time nevertheless expanded

inexorably. And there was Friedmann, working in obscurity.

It might have been thought that the demonstration that the Universe is indeed expanding would have given relativists a great fillip. The opposite seems to have been the case. People in the field were still struggling with the mathematical difficulties of curved space-time. On the other hand, empirically minded people took the line that general relativity would become a part of physics when there were meaningful ways in which it could be tested. They came along only in the 1960s. Even now, while general relativity has survived all the tests that have been made on it, such tests as there are are *local* tests. We await the detection of gravitational waves from anywhere, not to mention from the distant quasars. General relativity remains untested on the scale of the Universe, the structure it was designed to describe.

Of necessity, Tolman does not tackle one of the great surprises in the mathematics of cosmology — the recognition that in the Universe as it is now, the evidence suggests that space-time is hardly curved at all in Einstein's sense, but almost flat. But that is why the newer textbooks are able to use Newtonian gravitation for describing the Universe in the large.

General relativity is indispensable only when it is necessary to calculate the properties of black holes and the like, where the gravitational fields are intense enough to point the glaring gap in the present understanding of gravitation in the Universe: the sheer absence of a bridge between general relativity in Einstein's classical sense and the quantum mechanics that has made rational the rest of physics. People have been beating their heads on that problem for a quarter of a century. If it is not soon cleared up, there seems every likelihood that general relativity will once more lapse into disuse, as in the 1930s.

Meanwhile, there is an important lesson to be learned from this tale: the old books — Tolman's is more a tract than a textbook — are more interesting than their almost forgotten familiarity suggests. They are important not just for what they say, but for what they are obliged by ignorance to omit to say. Perhaps one of the many public bodies that now exist to improve the public understanding of science should commission a study to chart the evolution of understanding from a close reading of textbooks picked up in secondhand bookshops.

John Maddox

Acetylcholine revisited

Anders Björklund and Stephen B. Dunnett

If impaired cortical cholinergic function associated with damage to the basal forebrain cholinergic neuron system is instrumental in dementia-related cognitive decline^{1,2}, then restoration of forebrain cholinergic neurotransmission might be enough to improve at least some aspects of impaired learning and memory, particularly in conditions such as Alzheimer's disease. The neurotransmitter acetylcholine (ACh) is suspected to be an important participant in the maintenance of normal cognitive function, but it is not clear to what extent deficits seen after basal forebrain lesions can be attributed to loss of cortical cholinergic neurotransmission.

Recovery

Now, on page 484 of this issue, Winkler *et al.*³ show that, in rats, supplying ACh to the fronto-parietal cortex, achieved by transplantation of fibroblasts engineered to release ACh in a constitutive manner, can alleviate behavioural deficits induced by excitotoxic lesions of the forebrain cholinergic neurons located in the nucleus basalis magnocellularis (NBM). On the basis of their results, the authors propose that the presence of ACh in the neocortex could be essential for the recovery of spatial memory.

This study is of particular interest in that it is the first to use cells engineered by *ex vivo* gene transfer techniques to explore the role of cholinergic mechanisms in functional recovery after damage to the central nervous system. The results are important for two reasons. First, functional recovery was obtained by implants of cells that secrete ACh locally in a non-regulated, constitutive manner, whereas the NBM lesions are known to disrupt long-distance connections from subcortical areas and remove synaptic regulation of intrinsic cortical circuits. Second, recovery was seen after focal placements of the ACh cell transplants into the fronto-parietal cortex (six on each side), whereas the non-ACh-producing control cells had no effect. This observation, which is consistent with results from intracortical transplants of cholinergic neuroblasts from the fetal basal forebrain⁴⁻⁶, suggests that local ACh delivery to restricted areas of the neocortex may be sufficient to induce significant functional recovery in this model. These findings are pertinent to our understanding of the mechanisms of subcortical cholinergic regulation of cortical function: in particular, they suggest that cortical circuits may depend upon diffuse cholinergic activation to sustain their basic function, rather than upon temporally or spatially patterned inputs relayed via

afferent neuronal connections.

This brings us to what the new study³ can tell us about the normal role of the forebrain cholinergic system in cognitive function. Interpretation of the data of Winkler *et al.* is by no means straightforward in this respect. There is the question of specificity at three levels — whether the functional deficit induced by excitotoxic NBM lesions (and, by inference, in Alzheimer's dementia) is indeed cholinergic, cortical and cognitive in nature, which may not be the case. In fact, neither the lesion nor the behavioural task used in this study indicates a degree of specificity that would allow any firm conclusions.

Detailed analysis of the damage induced by injections of excitotoxic amino acids into the NBM has shown that both cholinergic and non-cholinergic neurons are affected and that ibotenic acid, the toxin used here, damages pallidal neurons as much as cholinergic neurons⁷. Indeed, more selective injury to NBM cholinergic neurons by quisqualic acid, AMPA or the saporin-192-IgG immunotoxin is associated with minimal deficits in the water-maze task, even though cholinergic denervation of the neocortex is more extensive than that caused by ibotenic acid^{8,9}.

The available evidence thus favours the view that the deficits in water-maze learning seen after NBM lesions in the rat are as likely to be attributable to damage in efferent pathways of the neostriatum as of cholinergic projections to the neocortex, and are as likely to involve some form of sensorimotor dysfunction or attentional disturbance as any impairment in cognitive function¹⁰. This issue needs a wider repertoire of behavioural tasks, as well as more specific cholinergic lesions, before it can be resolved.

We can conclude, therefore, that the behavioural deficits in NBM-lesioned rats may not be purely cholinergic, nor entirely cortical, nor cognitive in nature. But the available data indicate that local supply of ACh to the denervated cortex is sufficient to promote significant functional recovery in this model. Previous studies using transplants of developing forebrain cholinergic neurons from rat embryos were based on the idea that fetal cholinergic neurons may be able to substitute both structurally and functionally for the lost cholinergic afferents in NBM-lesioned animals. The new results suggest that impulse-dependent, regulated synaptic release may not be necessary for ACh to induce functional recovery.

In fact, Winkler *et al.*'s genetically engineered fibroblasts release ACh (and choline) in a non-regulated, constitutive manner. The fact that only the fibroblasts

bearing the choline acetyltransferase gene were effective does suggest that the observed effect was due to ACh release and activation of cholinergic receptors in the area surrounding the transplants. This is compatible with studies in which deficits associated with forebrain cholinergic damage are alleviated by cholinomimetic drugs such as physostigmine or tacrine. Moreover, non-cholinergic drugs that act to enhance cortical function in a more general way can provide a similar recovery of the behavioural deficits associated with NBM lesions and other types of cholinergic blockade^{11,12}. This suggests that a pharmacological reversal of deficits acting at the neocortical level can be mediated by as-yet rather ill-defined processes of 'cognitive enhancement', and does not necessarily indicate that the underlying deficit is itself cholinergic. An alternative explanation for the present results could be that, rather than acting as a replacement for the lost cholinergic innervation, ACh secreted by the engineered cell grafts acts in the host neocortex to promote the animal's ability to compensate for the lesion-induced injury.

Message

Although the mechanisms underlying behavioural recovery after brain damage are complex, this should not distract from the central message of Winkler *et al.*, namely that genetically engineered cells can be used to provide a local supply of biologically active molecules to an affected brain region in order to promote functional recovery and repair. This approach should provide new experimental tools for cell transplantation in animal disease models and offers promise for the development of prospective restorative therapies of brain damage and neurodegenerative disease. □

Anders Björklund is in the Department of Medical Cell Research, University of Lund, S-223 62 Lund, Sweden. Stephen B. Dunnett is in the MRC Cambridge Centre for Brain Repair, and the Department of Experimental Psychology, University of Cambridge, Cambridge CB2 2PY, UK.

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Colder, yet colder atoms

Christopher Foot

TECHNIQUES for cooling atoms have improved dramatically in the past few years, and recently Petrich *et al.* (*Phys. Rev. Lett.* **74**, 3352–3355; 1995) reached the lowest temperature so far — only 200 nanokelvin above absolute zero. This brings the possibility of seeing the long-sought Bose–Einstein condensation in a gas tantalizingly close. This development has created intense interest within the atomic physics community, because a gaseous Bose–Einstein condensation would be a new type of quantum system potentially manifesting many hitherto unknown phenomena.

At low temperatures the kinetic energy of atoms is small and they move slowly (for instance, velocities of the rubidium atoms in the experiment of Petrich *et al.* were only a few millimetres per second). These slow atoms do not behave like classical objects such as billiard balls but become more wave-like. This arises from the wave–particle duality in quantum mechanics which says that objects have a (de Broglie) wavelength, $\lambda = h/p$, which is inversely proportional to their momentum, p (h is Planck's constant). The wavelength roughly gives the distance over which the wavefunction of the atom is spread out; for example, in the new work the rubidium atoms were delocalized over $1\ \mu\text{m}$, which is many orders of magnitude greater than a typical 'atomic diameter' of a few ångströms.

In an atomic vapour that is sufficiently dense, the de Broglie wavelength can become comparable to the spacing between atoms so that their individual wavefunctions overlap. This makes it impossible to tell which atom is which (provided that they are all of the same type — rubidium-87 in this case — and in the same magnetic state). Quantum mechanics says that under these conditions identical particles are indistinguishable. Thus the cloud of atoms has a wavefunction that is coherent across the whole sample, so the whole system must be thought of as a macroscopic quantum object. For Bose–Einstein condensation, the particles must also be bosons (that is, obey Bose statistics; this is true for rubidium-87 because it has a total angular

momentum which is an integer multiple of $h/2\pi$).

The rubidium atoms in the experiment were collected from a low-pressure vapour in a small cell, and were initially cooled by the radiation force from laser light resonant with an atomic transition which exerted a strong damping force. However, wave–particle duality also ap-

plies to light, its particle-like nature being manifested as photons. Thus the radiation force is actually the result of bombarding the atom with many photons, and the minimum amount by which the atomic momentum can change is equal to the photon momentum — this discreteness limits the temperature.

To reach lower temperatures, Petrich *et al.* loaded the laser-cooled atoms into a magnetic trap produced by two coils with currents in opposite directions (Fig. 1). At first they used just the quadrupole magnetic field, which gives a point of zero field at the centre of the coils (midway along the axis between them), and the atoms congregate around this point of minimum magnetic potential energy. The trapping potential has a conical shape shown in Fig. 1. The sharp variation at the bottom of this trap near the field zero is not desirable, because atoms which pass very near to the centre 'see' a rapidly changing magnetic field direction. They cannot follow it, and therefore undergo (non-adiabatic) transitions to untrapped states.

Petrich *et al.* found that this loss mechanism was a severe problem and they invented a way to plug the leak. By applying a small magnetic field rotating in the horizontal plane, using two additional pairs of coils as shown in Fig. 2, they made the point of zero field (apex of the conical potential) move in a small circle about the trap centre. The atoms cannot follow this moving potential and, averaged over time, they see a smeared out potential which has a nicely rounded bottom. They called this a time-averaged orbiting potential (TOP) trap. It combines the tight confinement and convenience of a quadrupole trap with the advantages of having a harmonic potential and non-zero magnetic field at the bottom.

In this new type of magnetic trap, rubidium atoms underwent evaporative cooling to a temperature two orders of magnitude lower than the initial laser-cooled atoms. This works by allowing the most energetic atoms to escape, so that the average energy of those remaining decreases. Petrich *et al.* obtained the most successful evaporative cooling by applying a radio-frequency field to induce transitions for atoms with the highest potential energy to an untrapped state (see Fig. 2). Although this method decreases the total number of atoms, the density

actually increases because, as the temperature drops, the atoms sit lower down in the trapping potential. Finally, Petrich *et al.* obtained 20,000 atoms at 200 nK and a density of $6 \times 10^{10}\ \text{cm}^{-3}$. For Bose–Einstein condensation to occur at this density, the atoms would have to be cooled further to around 3 nK, corresponding to a de Broglie wavelength of a few micrometres. This could be possible using the new technique. Indeed, the

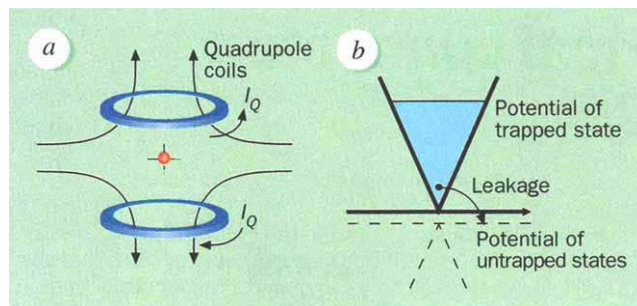


FIG. 1 *a*, The quadrupole magnetic field produced by two coils with equal and opposite currents. Atoms collect at the centre where the field is zero. *b*, The magnetic potential of the trapped state in a radial direction. Atoms moving near the sharp part of the potential at the bottom 'see' a rapidly changing field which causes transitions to untrapped states.

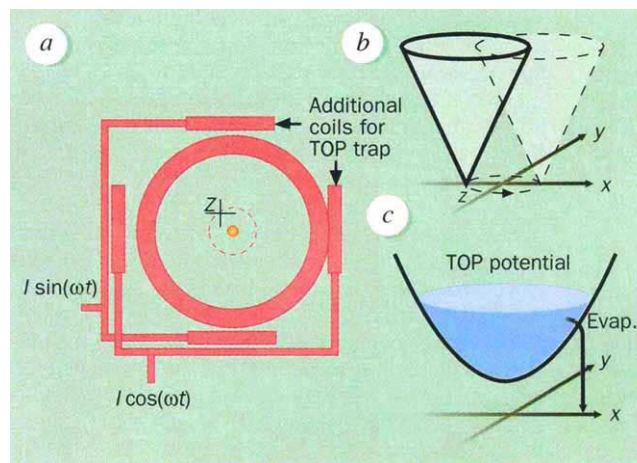


FIG. 2 *a*, In the new trap of Petrich *et al.* an additional rotating magnetic field is applied in the horizontal plane. The zero point (Z) rotates as shown. *b*, The potential of the quadrupole plus rotating field varies in time. The atoms see a time-averaged orbiting potential (TOP) as shown in *c*. *c*, The TOP trap has a harmonic potential with non-zero magnetic field at the bottom. Atoms are cooled in this trap by evaporation — removing atoms of above average energy followed by rethermalization through collisions.

plies to light, its particle-like nature being manifested as photons. Thus the radiation force is actually the result of bombarding the atom with many photons, and the minimum amount by which the atomic momentum can change is equal to the photon momentum — this discreteness limits the temperature.

To reach lower temperatures, Petrich *et al.* loaded the laser-cooled atoms into a magnetic trap produced by two coils with

same group has reported another order of magnitude improvement, down to 30 nK, at a recent conference.

A significant feature of this work is that the atoms did not stick together. The rates for various processes by which this could happen are not well known, and the observation that they do not occur opens the door for future work using alkali atoms.

If it is indeed possible to achieve Bose-Einstein condensation by evaporation in the TOP trap, which is considerably sim-

pler than other methods that are being attempted, then this new quantum state will become accessible for a whole new variety of experiments. We will at last be able to see the effects of Bose statistics in a clean way in such a gas, unaffected by strong interactions which dominate the behaviour in Bose systems such as superfluid liquid helium. □

Christopher Foot is in the Clarendon Laboratory, University of Oxford, Parks Road, Oxford OX1 3PU, UK.

GALAXY EVOLUTION

Radio and optical connected

George Helou

WITH instruments of striking diversity and precision, observational cosmologists translate the whispers of distant galaxies into clues to cosmic history. But the same corner of Universe is not often probed with more than one instrument, so we are left to piece together that history like the proverbial blind men examining an elephant. When two probes do intersect, the result is something akin to a Rosetta Stone, where observations at various wavelengths can be woven together to yield a hitherto unseen picture.

On page 471 of this issue¹, Windhorst *et al.* report on just such an intersection between a deep visible-light survey and a commensurately deep radio survey in one tiny, dark and carefully chosen patch of sky. From the comparison of radio and visible sources, and using a sprinkling of redshifts, the authors convincingly identify a mystery population of faint radio sources as disk galaxies undergoing profuse star formation at redshifts up to 0.5 and possibly beyond — that is, several billion years ago.

Starting a decade ago, deep radio surveys at gigahertz frequencies revealed source counts increasing faster than expected at flux levels below a millijansky (refs 2, 3). This increase pointed to a population distinct from the brighter sources, the classical 'radio galaxies', ellipsoidal conglomerates of old stars, which derived their radio power from the gravitational energy of matter accreting onto putative black holes at their centres. A strong case was made, albeit on indirect evidence, that the faint radio sources were distant objects similar to normal disk galaxies found in the local Universe. Disk galaxies have ample supplies of interstellar gas, and derive most of their radiated power from rapid nuclear burning in stars recently born of coalescing gas. Although the association between faint radio sources and disk galaxies was not controversial, the place of these galaxies in cosmic history, including their typical dis-

tance and luminosity, has remained a matter of debate.

Windhorst *et al.* used the Hubble Space Telescope (still in its aberrated state) for more than 10 hours to image a few square arcminutes of sky at sufficient resolution to reveal the morphology of any galaxies detected. They then observed the same field with the Very Large Array for 150 hours, synthesizing one of the most sensitive radio maps ever at 3.5 cm. A critical aspect of deep surveys is that sensitivity to weaker flux levels must be accompanied by finer spatial resolution, lest the more numerous weaker sources become indistinguishable and spoil the advantage of improved sensitivity. Matching exactly both sensitivity and resolution of instruments operating at very different wavelengths is nearly impossible, and one of the technical achievements of Windhorst and colleagues' study is that it edges close enough to such a match to make the comparison fruitful.

The study conclusively identifies faint radio sources as a population of disk galaxies. A surprisingly large fraction of these galaxies are distorted, suggesting that strong gravitational interactions are occurring, even perhaps severe disruptions or mergers. In the local Universe such morphologies are accompanied by episodes of massive star formation, but in the early Universe they may be a signal that galaxies are under construction⁴.

How actively, then, are these galaxies forming stars? With nearby disks as a guide, the radio data can be used to estimate the infrared emission from warm interstellar dust heated by the stars in each galaxy^{5,6}. For 90 per cent of the galaxies the estimated infrared luminosity exceeds the visible light output, in some cases by a hundred times or more. This is typical of the most active galaxies such as M83, M82 or Arp 220, with high-density interstellar media glowing brightly in intense 'starburst' episodes^{7,8}. The expected infrared fluxes would be fractions of a millijansky

near 60 μm , barely detectable by ESA's Infrared Space Observatory, which is due to be launched later this year. The Space Infrared Telescope Facility, under consideration by NASA, should detect such fluxes readily, though barely resolve them spatially. The combination of radio, infrared and optical emission-line data will allow a direct comparison of interstellar medium parameters — density, metallicity, dust-to-gas ratio, magnetic field strength, for instance — between these galaxies and their counterparts in the local Universe, a comparison that would measure evolution in galaxies and in their processes.

Redshift data place the brighter of Windhorst and co-workers' galaxies 1–5 billion light years away. Redshifts for the fainter galaxies can be obtained only with the largest optical telescopes but are necessary to complete the description of this sample, and ascertain whether these are more distant starbursts, or less spectacular galaxies at the same distance. A more complete picture would allow us to initiate a systematic tally of the formation of stars, and therefore of heavy elements, in the Universe since a redshift of 1. The result would then constrain the equivalent tally in the earlier Universe.

Besides the need for more redshifts, the visible-light survey would benefit from a follow-up with the aberration-corrected camera on the Hubble Space Telescope to look for lower surface brightness features or companions to these galaxies, and evaluate their relation to the fainter and presumably earlier objects recently reported by Dressler and colleagues⁴. On the radio side, it is important to clarify whether or not these are truly flat-spectrum radio sources, as earlier evidence weakly suggests. Nearby disk galaxies have sloped spectra that can be readily interpreted in terms of synchrotron emission from cosmic-ray electrons; flat spectra would call into question our understanding of the emission process in the distant galaxies.

Counting sources on the sky as a function of brightness is a fundamental test of cosmic geometry, one that is aimed at estimating how the volume of a sphere scales with its radius⁹. However, as more

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sensitive surveys at various wavelengths have reached deeper into distance and time, such counts have been mostly affected by the evolution of source populations with 'look-back' time. Evolution is thus a central element of cosmic history, and a prerequisite for the derivation of cosmic geometry^{9,10}. Unfortunately, the deep-count data do not adequately constrain the evolution models, and conclusions based on models have been

contradictory¹⁰. In this context, the study of Windhorst *et al.* stands out for its achievement of solid progress simply based on meticulous observations that drive our best telescopes to new heights of performance. □

George Helou is in the Infrared Processing and Analysis Center, California Institute of Technology, M/S 100-22, Pasadena, California 91125, USA.

BIOTECHNOLOGY

Making selection work

John F. Y. Brookfield

A POTENTIALLY happy and fruitful courtship is taking place between what, until now, have largely been two distinct disciplines in biology. The disciplines are population genetics and that loose consortium of pursuits known as biotechnology. The common interest is the use of selection to optimize the performance of drugs, antibodies and enzymes, and it brought representatives of the two sides together at a meeting last month*.

Evolution by natural selection consists of a mutational process creating genetic alternatives, of which that with the highest fitness increases in frequency. Mutation, selection and inheritance thereby successively increase mean fitness. Despite its lack of foresight, in that it selects amongst variants only on the basis of their present performance, and not their potential for subsequent improvement, the power of the process can be seen in the phenomenal success of living things.

Correspondingly, in the design of many new processes and products, the most effective strategy has not been rational planning on the basis of knowledge, but rather the creation of a selective environment in which products are successively optimized on the basis of a 'forced' process of evolution. We need to know how best to carry out such a strategy, by asking, for example, what is the optimal number of variants to screen, how many we should select, how much mutation we should impose on them, and whether we should, by recombination, bring together different favoured variants in the same replicating molecule. All of these questions are closely analogous to those of traditional population genetics and of computer-based genetic algorithms, hence the rationale for the meeting.

Most simply, forced evolution starts with blind mutation followed by screening, as exemplified by recent progress in combinatorial chemistry. M. Pavia

(Sphinx Pharmaceuticals) described methods for producing a vast range of small organic molecules, each of which can be tested for a variety of biological activities. For amino-acid sequences, the astronomical number of possible permutations requires successive rounds of optimization and mutation. In this way, the capacity of the enzyme subtilisin to function in the organic solvent dimethylformamide has been greatly improved (F. Arnold, California Inst. of Technol.). The experimenter still has to test alternative proteins, however, and retain the genes encoding the best.

More rapid selection is possible if molecules can be automatically retained as a result of their activities. Ribozymes can be developed from initial pools of RNAs of random sequence through the use of selection in which only those variants capable of ligating themselves to an anchored RNA are used to form the next generation (D. Bartel, Whitehead Inst.). Ribozymes, as with the hypothesized enzymatic RNAs in the 'RNA world'¹ at life's origin, carry their genotype with them, and selection of a molecule with enhanced activity also selects genetically. Evolution can only occur if the genotype is somehow coupled to the phenotype, as in early life where the move from an 'RNA world' to a 'ribonucleoprotein world' required the development of membranes so that only an improved RNA itself would receive the beneficial effects of the improved protein that it encoded. Libraries of antibodies displayed on phage (G. Winter, Laboratory of Molecular Biology, Cambridge) allow the coupling of protein phenotype and inheritance. A specific antibody displayed on the surface of the phage may bind its antigen; in this way, the attached phage, containing the genes encoding the antibody, is selected.

Notwithstanding the enormous power of forced evolution, participants at the meeting were reminded of the old story in which a tourist, seeking directions in an unfamiliar city, receives from a native the less-than-helpful advice: "Well, I

wouldn't start from here". The point is that an initially successful variant, although better than its competitors, might be even further from the overall optimum than was the starting point of the process. A primary determinant of the efficacy of forced evolution is the presence or absence of such epistasis. Will an advantageous genotype get stuck on an 'adaptive peak'? On such a peak, all single mutations are detrimental, although a major improvement would be produced by the right set of multiple changes.

In *Escherichia coli*, replicate populations, all initially of the same clonal genotype, were subjected to a new glucose-based environment^{2,3}. All replicates increased their fitness, but the phenotypes associated with this adaptation process differed. After 2,000 generations, the rate of improvement in fitness had dropped almost to nothing, despite continued mutation. This suggests that a range of possible mutations can adapt a genotype, but that, once some of these have occurred, subsequent mutations convey no advantage, despite their advantageousness in the genetic background of the initial clone. Genotypes thus evolve to different stable endpoints through the chance occurrence and fixation of different advantageous mutations⁴ — a phenomenon which, if general, would preclude the full explanation of organisms' phenotypes solely in terms of the selection imposed by their environments.

In applications of forced evolution, it seems that epistasis rarely traps molecules on adaptive peaks. Even if it does, strategies for escape are possible, such as recombination. A new method induces recombination *in vitro* through the use of primerless polymerase chain reactions with mixtures of randomly sheared molecules of different sequence^{5,6} (W. Stemmer, Affymax, Palo Alto). Again, close parallels are seen with population genetic explanations for the evolution of recombination.

Forced evolution will not be the solution to all problems in the design and optimization of new molecules and processes. But it may well be very helpful in tackling many of them, and in this the seemingly arcane field of theoretical population genetics may prove the most potent tool. □

John F. Y. Brookfield is in the Department of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK.

*Biotechnology and Biological Sciences Research Council Workshop on Forced Evolution, Warwick, UK, 15–16 May 1995.

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Keeping synapses up to speed

Pietro De Camilli

THE proper functioning of the nervous system demands that synapses should operate efficiently over a wide range of frequency stimulation. To guarantee neurotransmitter secretion during bursts of intense activity, nerve terminals store large pools of synaptic vesicles in reserve. Two reports published on pages 488 and 493 of this issue^{1,2} by Rosahl *et al.* and Pieribone *et al.* provide new insight into the mechanisms involved in the build-up of these reserves and reveal the consequence to synaptic physiology when they become depleted.

Depolarization of a nerve terminal induced by an action potential triggers exocytosis from a pool of ready-to-fuse synaptic vesicles — on average 10–20 vesicles at central synapses — which are docked at active zones of the presynaptic plasmalemma³. Membranes of fused vesicles are then reused for the generation of new fusion-competent vesicles by membrane recycling. But because this process is slower than the speed at which synaptic vesicles can be depleted from active zones during intense activity^{3,4}, presynaptic compartments are in danger of becoming rapidly depleted of synaptic vesicles during high-frequency stimulation unless new fusion-competent vesicles can be drawn rapidly to active zones from a reserve pool.

Synaptic vesicles are a special form of the endosome-derived vesicular carriers that are present in all cells (see *a* in the figure), so the basic mechanisms involved in exocytosis and recycling of synaptic vesicles are evolutionarily conserved⁵. However, only in the case of synaptic vesicles is exocytosis tightly regulated, implying that neuron-specific mechanisms are in operation. The protein synapsin is an abundant nerve-terminal specific protein which is thought to play a part in some aspect of the secretion process that is unique to synaptic vesicles^{6–8}.

Synapsin is a collective name for two very similar proteins, synapsin I and synapsin II, which were first identified in the 1970s by Greengard and co-workers as endogenous brain substrates for cyclic AMP and Ca^{2+} -dependent phosphorylation⁸. Synapsin I, but not synapsin II, contains a highly basic, proline-rich region at its carboxy terminus⁷. Synapsin is concentrated in nerve terminals, where it is peripherally associated with the cytoplasmic surface of synaptic vesicles. *In vitro* studies have shown that purified synapsin binds actin with high affinity, that it can crosslink synaptic vesicles to actin, and that its interaction with both actin and vesicles can be modulated by phosphorylation^{8,9}. Manipulations of nerve

terminals that enhance neurotransmitter secretion also induce an increase in the state of phosphorylation of synapsin. This change is paralleled by an increase in the amount of synapsin recovered in soluble fractions, suggesting that, *in vivo*, the interaction of synapsin with vesicles is dynamic⁸.

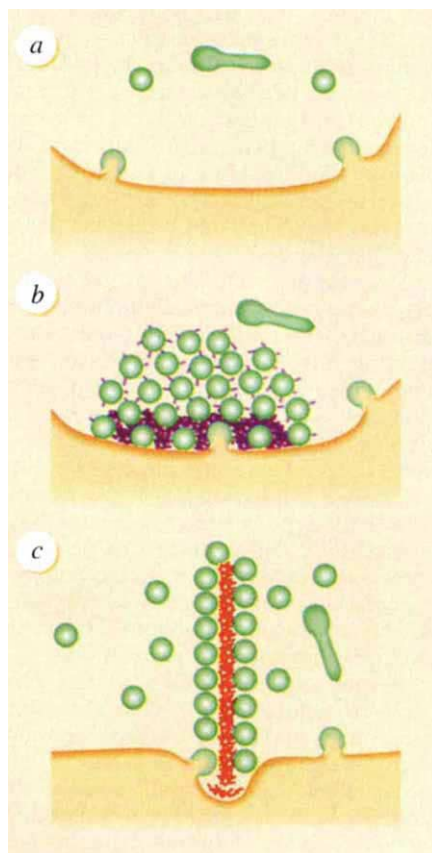
It has been proposed that synapsin provides a regulated link between synaptic vesicles and the cytomatrix of nerve terminals and that it may control the availability of synaptic vesicles for release. This would imply that the protein has a key role in both the ontogenesis and maintenance of synapses^{8,10}. It was therefore a surprise to find that mice lacking synapsin I displayed a relatively mild

phenotype, with no apparent change in a variety of functional and morphological parameters and only an altered paired-pulse facilitation (the phenomenon by which the second of two stimuli separated by a short interval elicits a greater release of neurotransmitter)¹¹. Although additional deficits have come to light in more detailed investigations of these mice^{1,2}, the synapsin I knockout results show that the function of synapsin I is dispensable for the basic process of neurotransmitter release at the synapse. The interpretation of these findings, however, is hampered by a possible redundancy between the functions of synapsins I and II.

Rosahl *et al.* have now generated knockout mice lacking synapsin II, or both synapsin I and II, and performed a thorough anatomical, histological and functional characterization of these animals¹. Even the double-knockout mice were viable, but they developed severe generalized seizures. Although the general architecture of the neuropile was unchanged, synaptic vesicle number and the level of intrinsic synaptic vesicle membrane proteins (but not of the corresponding messenger RNAs and of peripheral vesicle membrane proteins) were severely reduced. At hippocampal synapses, post-tetanic potentiation (an increased secretory response to a stimulus that follows a high-frequency train of action potentials) was drastically decreased and activity-dependent synaptic depression (the run-down of the secretory response during repetitive stimulation) was increased. Synapsin II-knockouts had a similar but less severely damaged phenotype, and generally the severity of the phenotype was roughly related to the number of missing synapsin alleles¹.

In an independent study, Pieribone *et al.* have tested the effect of the acute antibody-mediated disruption of synapsin function on the 'en passant' synapses of lamprey reticulospinal axons². The anatomical features of these axons make them a very elegant system for this type of study. The injection of antibodies directed against the conserved carboxy terminus of mammalian synapsins Ia and IIa, but not injections of control antibodies, caused a virtually complete loss of synaptic vesicle clusters at synapses, with the exception of the pool of vesicles close to the presynaptic plasmalemma. This morphological change did not affect the level of transmitter release elicited by low-frequency stimulation, but produced a pronounced reduction in release after high-frequency stimulation².

The results obtained in the lamprey are strikingly complementary to the results from the knockout mice. In both cases, disruption of synapsin function equals fewer vesicles at the synapse and, as a consequence, inability to maintain high levels of release during a burst of synaptic



Relationships between vesicular carriers of the housekeeping receptor-mediated recycling pathway (*a*), synaptic vesicles of conventional synapses (*b*) and synaptic vesicles of ribbon synapses (*c*). Fusion and budding mechanisms are thought to be very similar in all cases, but exocytosis is highly regulated in the case of synaptic vesicles. In addition to a small pool of ready-to-fuse docked synaptic vesicles, a large reserve pool of synaptic vesicles is present at synapses (*b* and *c*). It is proposed that the accumulation of this reserve pool involves synapsin at conventional synapses and the dense matrix of the ribbon at ribbon synapses.

activity. The more drastic phenotype observed in the antibody-injection studies and the different patterns of vesicle depletion (more global in the knockouts and very selective for the vesicle pool distal to active zones in the lamprey) may be at least partially explained by differences between the acute and the chronic disruption of a protein. In a chronic disruption, compensatory changes may come into play. In principle, the results of the two studies could be explained by defective recruitment of synaptic vesicles at presynaptic sites, or by impaired reuse, leading to increased synaptic vesicle catabolism¹. As disappearance of the vesicle cluster in the lamprey study was facilitated by, but not dependent on, stimulation of the synapse², Pieribone *et al.* take these data as strong support for the idea that synapsin is important for the recruitment of synaptic vesicle clusters at release sites.

A role of synapsin in trapping synaptic vesicles into a presynaptic matrix is consistent with several previous studies^{8,10} and with the mice knockout results. Inefficient retention of synaptic vesicles in nerve terminals may result in an increased turnover of intrinsic synaptic vesicle proteins. Peripheral membrane proteins that associate with synaptic vesicles only in nerve endings may be unchanged¹ because their accumulation at the axonal periphery is likely to be regulated by distinct mechanisms.

This putative role of synapsin is also in agreement with the absence of the protein from ribbon synapses of axonless cells of sensory organs¹². These synapses, which use synaptic vesicles that are otherwise very similar morphologically and biochemically to *bona fide* synaptic vesicles¹², have unique functional properties that correlate with special structural features. Neurotransmitter release is regulated tonically by graded changes in membrane potential, and high rates of synaptic vesicle exocytosis can be sustained for prolonged periods^{13–15}. At these synapses, synaptic vesicles are concentrated close to active zones by dense cytoplasmic bodies (which are ribbon-like in photoreceptor and bipolar cells of the retina) completely coated by synaptic vesicles. It was proposed that synaptic vesicle trapping mediated by the ribbon may guarantee a constant supply of vesicles during prolonged depolarizations^{13,15}. The dense body of ribbon synapses and a synapsin I-containing matrix may have corresponding functions at ribbon synapses and at conventional axonal synapses respectively (*c* and *b* in the figure).

It must be emphasized, however, that even these new studies do not allow unequivocal interpretations of synapsin I function, and other scenarios are possible. For example, changes in paired-pulse facili-

itation (a phenomenon that occurs on a scale of milliseconds) observed in synapsin I (refs 1, 11), but not in synapsin-II or double-knockout mice¹, may indicate a specific role for synapsin I very proximal to fusion. On the other hand, this effect may be indirect and mediated by proteins that normally interact with the proline-rich carboxy terminal region of synapsin I (for example SH3-containing proteins¹⁶). In the synapsin-deficient mice, the selective increase in Rab5, a protein thought to be involved in the endocytic pathway¹⁷, cannot yet be explained.

Irrespective of the interpretation, the

phenotype induced by synapsin disruption offers fresh insights into how synapses work and shows that a key function of synapsin is to ensure a steady supply of fusion-competent vesicles to release sites. Synapsin plays a fundamental role in conferring upon synapses the plasticity required for the complex functions of the nervous system. □

Pietro De Camilli is in the Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 07510, USA.

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TECTONICS

Upwardly mobile hot crust

Simon L. Harley

LARGE-scale geological features such as mountain belts and continental rift zones are a reminder of the advances made in understanding the Earth through plate tectonics. But they also bring into focus the very real limitations on present knowledge of the deeper-level processes that accompany large-scale continental deformation. Quantification of the timescales of thickening, heating and thinning of the continental crust in response to magmatism or plate tectonic processes is often very difficult, and integration of time with the metamorphic record preserved in rocks that have 'seen' the tectonic history remains an elusive goal.

The problem of inserting timescales into this geological equation is nowhere more acute than in unravelling the often complex histories recorded in granulite terrains. These are high-temperature (>750 °C) metamorphic belts, which many geologists would argue form in the deeper parts of continental rifts but also comprise the roots of mountain belts formed through continental collision, such as the Alps and Himalayas, as well as magmatic arcs. How rapidly, and at what stage of a crustal thickening–thinning cycle, do the high-temperature granulites form in the deep crust (15–50 km)? How fast, and by what mechanisms, does a hot and probably overthickened continental crust evolve to approach thermal and gravitational equilibrium?

Gibson and Ireland (page 479 of this issue¹) marshal geochronological and metamorphic data from a comparatively young belt of 'hot rocks' — the 110-million-year-old Fiordland granulites of New Zealand — that convincingly demonstrate a rapid evolution to high-grade conditions at a depth of 40 km, followed by further granulite metamorphism that progressed during exhumation to 20 km depth. This history of crust formation, metamorphism and partial unroofing is telescoped into a 20–30-million-year interval, terminated by cooling that occurred only after the rapid exhumation.

The authors also made further observations on deformational fabrics associated with the high-temperature metamorphism, grade changes across ductile shear zones, and correlations with a higher-level but coeval metamorphic core complex now exposed further north along the later Alpine fault. They use these data to argue in favour of the plausible model that initial exhumation of the deep crust and formation of new granulites were accomplished through extensional collapse of magmatically thickened continental crust in the Early Cretaceous.

The Fiordland granulite history is not unique, in that other high-grade metamorphic terrains exhibit similar pressure–temperature conditions and evolutionary paths. But the unambiguous time framework provided by Gibson and Ireland is

important, given present controversies about the significance of pressure-temperature paths^{2,3} and uncertainties emerging over the interpretation of isotopic dating systems^{3,4} in other high-grade terrains.

Most of the 'hot rocks' known as granulites are exposed as vast areas of the old shields of continents. Some, however, as at Fiordland, are fortuitously presented for inspection in younger mountain belts where their recent tectonic contexts can be readily evaluated and their tectonic significance better assessed. Many granulites, ancient and recent, preserve in their mineral assemblages, and often in delicate microscopic reaction textures, a detailed record of their pressure-temperature evolution that can be used to track their depth-temperature evolution with time, and therefore that of the crust.

Among the many possible post-peak metamorphic pressure-temperature paths are two end members: cooling without much change in crustal depth, otherwise labelled near-isobaric cooling (IBC); and exhumation or unroofing through significant crustal depths without much cooling, or near-isothermal decompression (ITD). Current thinking is that such ITD has to be rapid — on a timescale less than that of thermal relaxation by conduction. So fast exhumation mechanisms acting over sufficient timescales (10 million years) are invoked to explain ITD where it is clear that the history is indeed linked to a single metamorphic cycle. It is not surprising, then, that extensional collapse of thickened crust or continental extension accompanied by magmatism have become fashionable models for the formation and evolution of granulites.

Gibson and Ireland go further than simply relating a granulite metamorphism to rapid extension. They postulate that the Fiordland granulites may typify what the deeper and unexposed levels of an active extensional region such as the Basin and Range province of the United States might look like, at least in terms of the deformational structures and metamorphic grades. The extensional unroofing proposed for the Fiordland granulites is indeed similar to the exposure of core complexes; however, analogies with the Basin and Range extensional province should be viewed with some caution as the geometry of extension in the Fiordland case may differ from the possible geometries for the Basin and Range. The comparisons proposed by Gibson and Ireland should promote further structural and kinematic studies of granulite terrains that centre on the question of how extension is actually accommodated at such depths and temperatures.

Analysis of the thermal history of deep crustal rocks is not straightforward. Because of the high temperatures involved and a reliance on temperature-dependent

isotopic systems⁴, it has seldom proved possible to define timescales of decompression precisely enough to show that the exhumation rates were necessarily more rapid than those that could be attained simply through erosion. Likewise, linking extensional deformational structures with the pressure-temperature history, and tying both to a well-defined time framework, has proved difficult for many older but important granulite terrains⁵. These older belts have often had complex histories, including the superposition of several unrelated events. Indeed, it has been argued by several workers that an ITD history deduced principally from metamorphic reaction textures may only be apparent — the result of partial overprinting of an earlier thermal event by a later, lower-pressure one, separated from the first by a considerable time^{2,3}. Resolution of this problem clearly requires high-quality isotopic dating, and the Fiordland example shows it can be done, and conclusively.

Can most granulites be linked to rapid extension following previous thickening events, or does rifting alone account for granulite metamorphism? Is the core complex analogy proposed for Fiordland¹ appropriate to other granulites in which extensional structures are observed? These are complex questions for which simple and general answers are not likely. High-grade metamorphic terrains not only preserve a variety of pressure-temperature histories, including the ITD and IBC styles, but also a range of apparent cooling and exhumation rates. For example, recent geochronology has shown that extensional unroofing of the deep granulites of the Grenville province was a slow process involving shear zones active for some 150 million years⁵. This slow exhumation contrasts with the rapid unroofing typical of core complexes⁶ and apparent in the Fiordland case¹, and implies different tectonic processes controlling the post-granulite crustal evolution. The Fiordland study presented by Gibson and Ireland should be regarded as a stimulus for further studies of deep-crustal granulites, rather than a template for the interpretation of these important metamorphic terrains. □

Simon L. Harley is in the Department of Geology and Geophysics, University of Edinburgh, West Mains Road, Edinburgh EH9 3JW, UK.

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DAEDALUS

Water everywhere

EVERY cubic metre of air contains about 10 g of water. Shipwrecked mariners and desert dwellers, and householders resentful of the cost and contamination of their piped supply, might welcome a way of extracting it. Some chemical absorbents, such as silica gel and calcium chloride, extract water from the air very efficiently. This, however, merely generates a new problem: how to extract the water from the absorbent? Daedalus now has a cunning answer.

It is based on those 'super-slurper' polymers, widely used in nappies and bandages, which absorb hundreds of times their weight of water. They don't work by capillary action like a towel; they imbibe water chemi-osmotically and are plasticized by it. They can even take up water from the air, though current nappy formulations are designed to minimize this effect. Daedalus, however, wants to maximize it. DREADCO's chemists are working on a super-slurper to absorb vast amounts of water from the air. And how to extract the water from this new product? Easy, says Daedalus: you eat it.

Many super-slurpers are almost edible as it is. They are copolymers of corn starch with a certain amount of acrylic monomer. The DREADCO team are adding polymerizable amino acids to the recipe, seeking an edible and hygroscopic super-slurper. Just leave the product in the air — ideally overnight, to take up water vapour from the colder and more humid night air — and it will swell into a flabby gel containing up to 99% absorbed water. You can then have it for breakfast. Your digestion will break it down simply and safely. Its starch and amino acids will have some value as food; the acrylic residues will become healthy, inert fibre. But its main component, water, will simply be released in your stomach. In effect, it will be a solid drink.

'Drink Me', as the new product will be called, will be snapped up by explorers, sailors and tourists suspicious of the local water. About 10–20 g a day will save them from having to drink anything at all. Tea, coffee and orange flavours will be available. Drink Me could even be used for washing. Rolled over the skin, it would act rather like those sticky rollers which remove fluff from clothes.

Drink Me may compete in the slimming market, too. In its swollen state it will be the bulkiest, lowest-calorie food imaginable. Even better, a consumer who ate it dry, and then drank a hundred times as much water, would experience such a sudden massive bloating of the stomach that he would not want to eat anything else for some time.

David Jones

Is thalidomide mutagenic?

SIR — McBride¹ has suggested a mutagenic mechanism to account for two instances of men with thalidomide-induced deformities having a deformed child among normal offspring. This suggestion was immediately challenged²⁻⁴ on genetic and morphological grounds. Read considered the association unfounded² and Smithells³ specifically cautioned that the McBride hypothesis was without any scientific foundation and should not be allowed to stir up inappropriate anxieties. However, the matter has been taken up by several newspapers, in particular by the UK *Daily Express*, in whose 4 May edition Swards and Fuller⁵ speculated on a possible third-generation effect. The status of thalidomide as an experimental mutagen was not discussed in any of these articles.

Despite a few early, sporadic reports of the possible mutagenicity of thalidomide to onion root tips and mosquito larvae, most of the limited published literature concerns its non-mutagenicity. Negative *Salmonella* mutation data have been reported by Gordon⁶ derived from experiments using a range of metabolizing tissues taken from species sensitive to the dysmorphic action of thalidomide. In a particularly relevant study, Soukup *et al.*⁷ reported the absence of an increase in chromosomal aberrations in rat and rabbit embryos exposed to thalidomide *in utero*. However, Roux *et al.*⁸ observed positive effects in similar experiments, noting the irregular results that thalidomide often gives in such assays and recommending further studies.

The situation was clouded at an early stage by a curious exchange that appeared in *New Scientist* in 1983. MacKenzie⁹ wrote an article entitled 'Secret tests say thalidomide is mutagenic'. The unpublished work of Hagström was claimed to show that thalidomide is mutagenic to *Salmonella* and to the mouse bone marrow. The German manufacturers of thalidomide, Grünenthal, contested this and stated, again in the absence of data, that thalidomide was negative in the *Salmonella* assay, in a mouse bone-marrow micronucleus assay and in a mouse germ-cell mutation assay¹⁰. The negative germ-cell result is pertinent to the current debate and is consistent with earlier data¹¹.

At that point (1983) we decided to eval-

uate the mutagenicity of thalidomide ourselves. We observed negative results in extensive *Salmonella* and mouse bone-marrow assays. Several colleagues from other laboratories also contributed negative data from assays using yeast, fruit flies and grasshoppers. Being consistent with the claims made by Grünenthal, those negative data were not published. However, given the recent interest in this

matter we are currently repeating and extending those earlier studies and will publish them in the near future. Pending that, and a formal review of the status of thalidomide as an experimental mutagen, public discussion of McBride's paper¹ should include reference to the responses made to it by Read, Smithells and Kida.

J. Ashby

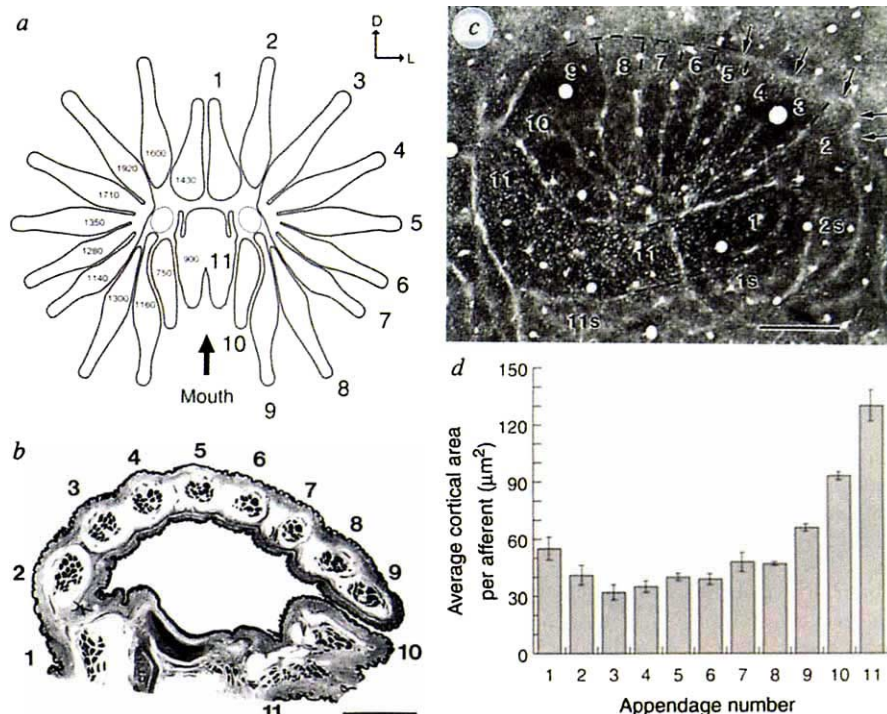
H. Tinwell

Zeneca Central Toxicology Laboratory,
Alderley Park,
Cheshire SK10 4TJ, UK

Magnified cortex in star-nosed moles

SIR — The mammalian cortex contains orderly maps of sensory surfaces with disproportionately large representations of behaviourally important areas. Are these enlarged areas proportional to their peripheral innervation densities? In the somatosensory system of rodents, the size of each cortical barrel is directly proportional to the innervation density of the corresponding whisker¹. It has long been debated whether the large cortical representation of the retinal fovea is similarly a

reflection of the increased density of ganglion cells in the fovea^{2,3}, or is larger than would be predicted from ganglion cell densities alone⁴⁻⁶. This question is addressed here by comparing the number of afferents from the nasal appendages of the star-nosed mole (*Condylura cristata*) with the extent of their corresponding cortical representations. The nose of this mole consists of 22 fleshy appendages (see figure), each represented in the cortex by a band visible in cytochrome oxidase



a, Diagram of the mole's nose as viewed from the front. Appendages are numbered 1-11. Left side, number of touch receptors, called Eimer's organs, located on each appendage from a single specimen. Note that the 11th appendage has relatively few sensory receptors on its surface. b, A 1-μm section taken caudal to the appendages reveals the 11 nerve branches from the 11 appendages from one half of the nose. There are approximately 50,000 myelinated afferents innervating each half-nose (roughly 4,000-5,000 per appendage). Bar, 1 mm. c, Tangential section of flattened somatosensory cortex, stained with cytochrome oxidase, reveals the representation of the 11th appendage. Note the huge representation of the 11th appendage (more than 25% of the nose field). Arrows, septa at the border of the nose field. Bar, 500 μm. d, Average cortical area per myelinated afferent for each appendage of the nose (means from four animals). The area of cortex devoted to each appendage is not proportional to the corresponding peripheral innervation density. Appendages 9, 10 and 11, which form an opening in front of the mouth, also have the highest cortical area per fibre. Bars, s.e.m.

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stains⁷. Thus, the amount of cortex devoted to each appendage can be accurately determined.

To quantify the sensory input from each appendage, one half of the nose from each of four moles was thin-sectioned at a level where a single nerve branch was visible for each appendage (*b* in the figure). Each nerve branch was photographed and the myelinated fibres in each were counted. The cortex from the corresponding hemisphere of each mole was flattened, sectioned tangential to the surface at 60–90 mm, and stained with cytochrome oxidase to reveal the 11 bands representing each half nose (*c*).

The sizes of the cortical representations of the nasal appendages are not proportional to their peripheral innervation densities (*d*). The 11th appendage, which is preferentially used to explore objects, is greatly over-represented in cortex, occupying more than 25% of the nose field. In addition, appendages 1, 9 and 10, which are also used during feeding⁸, show disproportionate cortical expansions. The 11th appendage is allocated approximately 130 μm^2 of cortex per afferent, whereas most of the other appendages are allocated between 30 and 50 μm^2 per afferent. Similar results have been obtained for the visual system in rhesus monkeys, where ganglion cells in the retinal fovea occupied 3.3–5.9 times more cortical area than ganglion cells in the peripheral retina⁴.

These results are the first report of a disproportionately expanded cortical rep-

resentation of peripheral inputs in the somatosensory system and they raise important questions about the development and maintenance of cortical areas. Although the results are consistent with the findings in the somatosensory cortex of primates, where additional magnification of cortical areas occurs in response to increased peripheral stimulation⁹, the expanded areas in the mole somatosensory cortex are not the result of an experimental manipulation. Because of the long evolutionary history of this functional fovea, we could ask whether there are phylogenetic contributions to this cortical specialization, or whether the preferential cortical magnification is simply the result of the regional differences in patterns of stimulation across the sensory surfaces during ontogeny (and thereafter). This question might be addressed in neonatal star-nosed moles.

Kenneth C. Catania

Department of Psychology,
Vanderbilt University,
Nashville, Tennessee 37240, USA

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Sex of the last wild Spix's macaw

SIR — The Spix's macaw (*Cyanopsitta spixii*) is the world's most endangered bird¹. Only 32 now survive and since 1987 only one individual has remained in the wild. Coupled with a programme of releases, it is hoped that this individual will help found a new wild population. Unfortunately, the Spix's macaw, like most bird species, is sexually monomorphic. Although behavioural observations suggest that the wild bird is male, this has never been verified. It is essential to have this information if a prospective mate is to be released.

Confirmation of the sex of the wild Spix's macaw would normally involve its capture, but this course has been ruled out as a significant risk to the bird. The only apparent alternative would be to use a test based on the polymerase chain reaction (PCR)² to identify the sex of this individual from the DNA traces present in moulted feathers³. Unfortunately, no such technique has been described applicable to this, or indeed any other, bird species.

Birds exhibit female heterogamety: that is, females have one W and one Z sex

chromosome whereas males have two Z chromosomes. Thus, the presence of a W-unique genetic marker is diagnostic of the female sex. We recently described a technique to isolate sex-specific genetic markers⁴ and have used this method to identify a highly conserved gene (*C-W*) from the chicken which seems to be W-linked in all birds, with the possible exception of the primitive ratites (our unpublished data). A second closely related gene (*C-2*) is situated elsewhere in the genome.

Stratagene provided a genomic library from the closely related hyacinth macaw (*Anodorhynchus hyacinthinus*), and from this we isolated a homologue of *C-W* and designed PCR primers to amplify a 104-base-pair (bp) region of both *C-W* and *C-2* from Spix's macaw DNA. Sequence determination revealed that the *C-W*-derived PCR product possessed a *DdeI* restriction enzyme site which was absent in the *C-2* product. Thus, PCR amplification and *DdeI* cleavage of male Spix's macaw DNA yields only a single product of 104 bp, whereas from female DNA two products are apparent, one of 104 and one of 73 bp. The presence of the *C-2* product in both



DdeI-restricted PCR products showing that the remaining wild Spix's macaw is male. Lane 1, wild bird; 2, negative extraction control; 3, known male; 4, known female. The larger fragment is 104 bp long and the female W-chromosome-specific fragment is 73 bp.

METHODS. DNA from the wild bird was extracted⁵ from 1-cm portions of the tips of 3 moulted flight feathers collected over the past 2 years. The negative extraction control was taken through an identical procedure. 1.5% of these extraction products or 50 ng genomic DNA from the reference samples were subjected to semi-nested PCR. Primary amplification consisted of 20 cycles with primers (5' to 3') P3, AGATATTCGGGATCTGATAGTGA, and P2, TCTGCATCGCTAAATCCTTT; 1% of the primary PCR product was subjected to 30 cycles of amplification with P2 and P1, ATATCTGATCTGATAGTGA(C/T)TC. Samples were denatured for 1.5 min at 95 °C, then cycled between 57 °C per 30 s, 72 °C per 15 s and 94 °C per 30 s with a 5-min final extension. Products were precipitated, cut with *DdeI*, reprecipitated and electrophoresed through a Visigal separation matrix (Stratagene). All appropriate precautions and negative controls⁵ were implemented. The accuracy of the test was confirmed using DNA from Spix's and hyacinth macaws of known sex ($n = 5$, $P = 0.03$). Uncut secondary PCR product from the wild bird was isolated⁶, cloned using the Stratagene pCR-Script SK(+) kit and sequenced (Amersham: 7-deaza-dGTP kit) to confirm that the product had originated from a Spix's macaw. We thank Stratagene, M. Kelsey, P. W. H. Holland and J. R. Krebs for advice and assistance. C-W and C-2 are subject to patent application 9413821.1.

sexes acts as a control to ensure the PCR amplification has been successful.

We extracted DNA from feathers moulted by the wild Spix's macaw using a technique devised to purify ancient DNA⁵. The PCR-based test described above was used to demonstrate that *C-W* was not present in the sample (see figure). This confirmed that the wild bird is male. A female Spix's macaw has recently been released as a prospective mate.

Richard Griffiths

Bela Tiwari

Ecology and Behaviour Group,
Department of Zoology,
South Parks Road,
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Scientific approach to ski safety

SIR — During the 1994 Ski Flying World Championships in Planica, Slovenia, athletes performed jumps of more than 200 m for the first time, the present record being 209 m. Despite the fact that millions of spectators witnessed these jumps, distances of more than 191 m have been ignored by the International Ski Federation (FIS) in an attempt to reduce the jump lengths; this procedure obviously does not reduce the hazards of this dangerous sport. Scientific studies are urgently needed to provide a reliable basis on which safety enhancement strategies can be based.

We have performed wind-tunnel measurements with world class athletes in various flight positions, undertaken field measurements during the world championships in ski flying 1994, and have mapped ski jumping to a computable simulation model. Our results explain the effects of equipment, flight style changes, the reason for the high tumbling risk and sensitivity to gusts observed. This information is of use for making changes to the FIS regulations.

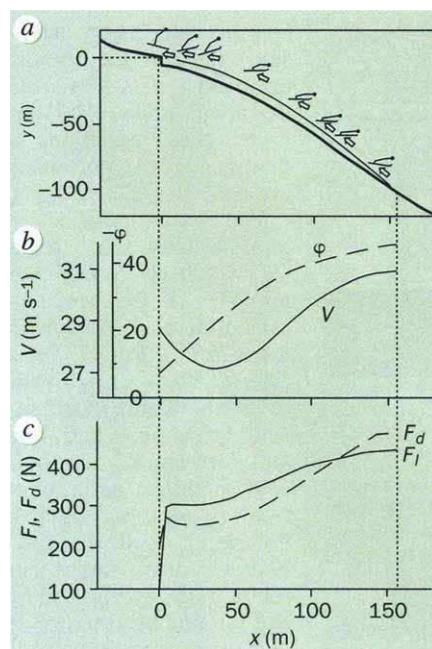
We measured the high torque of the skis in the airstream and the FIS followed our suggestion to limit the percentage of front ski to total ski length before the 1994–95 winter (to 57%). As a consequence the pitching moment balance has been eased and only one tumbling accident occurred during the 1994–95 World Cup, compared with 10 in 1993–94. Another urgent problem is the anorexia deliberately induced by the athletes (low weight increases the jump length). We suggest a regulation relating ski length to body weight (a self-regulating approach).

Simulations can predict the trajectory during the flight phase and investigate the effects of parameter and initial value variations^{1–4}. We developed a highly reliable mapping of ski jumping to a computable simulation model, which contains the dependencies of the lift and drag forces to the flight position. A representative set of data, obtained with the 1994–95 World Cup winner (who was the first to fly more than 200 m) in a 5 x 5 m² wind tunnel, was used to determine a reference jump. The values for the lift area L and the drag area D from the series of wind tunnel measurements were chosen according to the mean position angles measured for 15 excellent jumps during the world championships in ski flying, Planica, Slovenia, 1994.

L and D values have increased markedly during the past two decades⁵ due to changes in equipment and flight style. L and D measured with the holder of the

world record in 1976 using his old equipment and flight style were less than 60% of the results obtained with athletes of today.

The figure (a) shows the profile of the jumping hill in Planica and the trajectory $y = y(x)$ of this reference jump. The approach velocity v_0 was set to 28.6 m s⁻¹ (mean of the officially measured values). The jump length of $l = 186.7$ m to be obtained (mean jump length from the field) was found at a take-off velocity component due to the athlete's jumping force (perpendicular to the ramp) of $v_{po} = 2.24$ m s⁻¹. The flight path deviates remarkably from the parabolic trajectory. The actual angle of the tangent to the



Results with the reference jump. a, Profile of the jumping hill in Planica and the trajectory $y(x)$, with the flight position angles found in the field (means of 15 jumps) sketched above. b, Velocity $v(x)$ and angle of the flight path $\phi(x)$. c, Lift force F_l and drag force F_d . L and D values used for this reference jump (in m²): 0.2 and 0.4 (at $t = 0$ s), 0.65 and 0.60 (0.2 s), 0.68 and 0.58 (0.4 s), 0.77 and 0.64 (2.3 s), 0.79 and 0.73 (4.0 s), 0.78 and 0.79 (5.0 s) and 0.79 and 0.86 from 6.0 s onwards. Linear interpolation was used between. The air density was 1.15 kg m⁻³; the mass m of the athlete with equipment was 70 kg.

path $\phi = \phi(x)$, and the velocity $v = v(x)$ are shown in b. The plot in c of the lift force F_l (acting perpendicularly to the actual tangent to the path) and the drag force F_d shows that the velocity component perpendicular to the hill at landing (4.18 m s⁻¹) corresponds to that of a jump onto a horizontal plane from $h_l = 0.89$ m height (equivalent landing height).

How do the initial values and parameters influence the flight? We kept L and D values constant from $t = 5$ s onwards in

the following simulation. The jump length l obtained this way is 186.6 m, the flight time $t_f = 6.59$ s, the landing velocity $v_l = 31.4$ m s⁻¹, and the equivalent landing height $h_l = 0.89$ m (at $v_0 = 28.6$ m s⁻¹, $v_{po} = 2.24$ m s⁻¹, $m = 70$ kg). Starting out from this simulation, an increase of v_{po} to 3.0 m s⁻¹ (variation A), corresponding to an excellent take-off jump of the athlete⁴, increases the jump length to 195 m ($t_f = 6.92$ s, $v_l = 31.52$ m s⁻¹, $h_l = 1.59$ m). The same jump length can be obtained using an approach velocity v_0 of 28.98 m s⁻¹ (B). A wind blowing up the hill (130°, measured from a horizontal line) with a constant speed of 1.4 m s⁻¹ also results in $l = 195$ m (C). We also considered variations of the L and D values in functions corresponding to different flight styles, athletes or equipment. Increasing all L and D values in these functions by 11% (case D) or just L by 2.3% (E) also leads to a 195-m jump. A reduction of the mass (athlete with equipment) to 63 kg also results in $l = 195$ m (F). Finally, a shallow ramp angle of -9.7° (compared with -11.6°) yields $l = 195$ m again (G). In simulations C, D, E and F the equivalent landing height is reduced to 1.37 m, compared with A (1.59), B (1.49) or G (1.62 m).

In the real world, the change of one parameter will influence the others; athletes have to solve extremely difficult optimization problems in real time. The longest jump performed so far led to 209 m, which results from a simulation using $v_0 = 28.44$ m s⁻¹ (measured in the field), $v_{po} = 3.0$ m s⁻¹ (assumption of an excellent take-off jump)⁴, and L increased by 5.6%. This increase in L is realistically imaginable for this world-record jump. The landing velocity obtained is 31.44 m s⁻¹ and $h_l = 2.43$ m. Using an appropriate parameter protocol to simulate the 196-m jump from 1995 at the Oberstdorf jumping hill results in $h_l = 2.62$ m. The athlete could not stand this jump in the competition.

Wolfram Müller

Dieter Platzer

Bernhard Schmöler

*Institut für Medizinische Physik und Biophysik,
Harrachgasse 21,
8010 Graz, Austria*

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

Delivery of a nematocyst toxin

SIR — Cnidaria (hydras, jellyfish, sea anemones and corals) possess a wide array of neurotoxic, cytolytic and enzymatic¹ substances. Despite the fact that these toxic substances were isolated mainly from homogenates of entire animals or tentacles, their storage and delivery sites are attributes to nematocysts, the stinging subcellular organelles. Nematocysts consist of a capsule containing a highly folded eversible tubule². The discharge of the nematocysts is driven by

the capsule's high internal hydrostatic pressure of 15 megapascals (≈ 150 atmospheres), which causes the eversion of the tubule with accelerations up to 40,000g and therefore comprises one of the fastest events in biology^{3,4}. However, the ultrastructural and mechanical data do not explain toxin allocation and the mode of their delivery. Further, even the notion that nematocysts serve as the venom source was not unequivocally established.

The present study aimed to establish the chemical nature of jellyfish toxins, their subcellular localization and the route of their delivery. We show that cnidarian venom is stored in nematocysts on the outer surface of the tubule and is delivered upon its discharge (eversion–extension) through the spirally arranged array of the extended hollow tubular barbs, thus resembling a multiheaded poisonous arrow.

We have purified a phospholipase A₂ toxin (M_r 16,000; ref. 5) from the tentacles of the Mediterranean medusa *Rhopilema nomadica*. Purity was assessed by electrophoresis (*a* in the figure), amino-acid analysis and by amino-terminal amino-acid sequence determination (*g* in the figure). Polyclonal antitoxin antibodies were raised in rabbit⁶, affinity-purified by slot immunoblotting (*b*, *d*) and used as immunocytochemical tools to study toxin allocation by light (*e*, *f*) and electron microscopy⁷. Immunoblot revealed the presence of the toxin in the fishing tentacles, but not in the bell tissues (*b–d*). The bell, in contrast to the tentacles, is devoid of nematocysts. The toxin may therefore be attributed to the venomous hunting apparatus of the jellyfish, and is not likely to be a widely distributed defence chemical.

Immunocytochemical staining of cryostat-sliced nematocysts revealed a highly specific allocation of the toxin limited to their tubular segments (*e*, *f*). In the resting nematocyst (*e*) the capsular lumen and wall were devoid of immunofluorescent staining. The allocation of the

toxin on the discharged tubule revealed a helical shape (*f*) which parallels the helical arrangement of the barbs. The latter observation has raised the possibility that the hollow barbs⁸ may function as a device for toxin delivery. This essential aspect was further studied by high-resolution immunogold technique and electron microscopy⁷. It has been shown that the toxin is externally allocated on the surface of the inverted–undischarged tubule. In the everted–discharged tubule the toxin occurs on its internal surface and accumulates in the vicinity of the bases of the hollow barbs and their lumina (data not shown).

The above data provide a visualization of the route of toxin translocation and release schematically presented in *h*. In the resting nematocyst, toxin is located in the crypts (invaginations) of the outer surface of the twisted, folded and inverted tubule, the lumen of which is occupied by the internalized barbs. During discharge and tubule eversion toxin is translocated from the outer to the inner surface of the everted and extended tubule while aggregating near the bases of and within the hollow barbs during release.

We assume that the hydrostatic pressure raised in the dischargeable capsule⁹, causing the tubule eversion, also supplies the appropriate propulsion of toxin delivery through the barb system. This notion is supported by a study showing that the fibre-like structure of the capsule's inner wall, which provides the tensile strength necessary to withstand the capsule's high osmotic pressure, continues along the tubule's wall³. Thus, the nematocyst delivery system may provide an example of subcellular protein translocation based on simple mechanical forces.

A. Lotan*

L. Fishman*

Y. Loya†

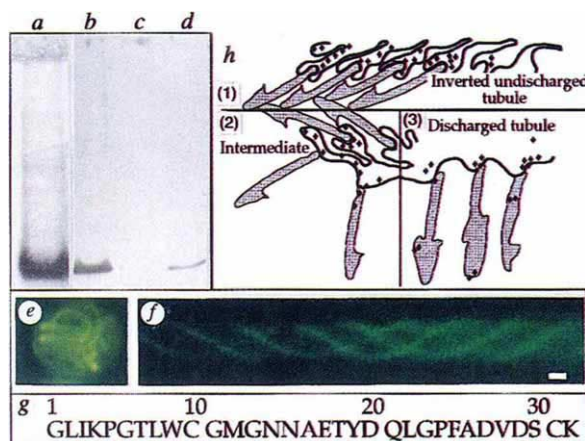
E. Zlotkin*

**Department of Cell and Animal Biology, Life Science Institute, Hebrew University,*

Jerusalem 91904, Israel

†Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

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Chemistry, location and delivery of a nematocyst toxin derived from *R. nomadica*. **a**, SDS–PAGE electrophoresis of 2 μ g toxin. **b–d**, Immunoblots. Separation of 10 μ g proteins extracted from the fishing tentacles (*b*), bell tissues (*c*) and 0.1 μ g of purified toxin (*d*). Tentacles of the freshly collected jellyfish were cut at sea, stored over dried ice, then deep-frozen (-80°C). Tentacles were thawed at 25°C followed by centrifugation ($15,000g$, 30 min). The supernatant-containing material from discharged nematocysts was desalted by dialysis and separated by DEAE–cellulose anion-exchange column followed by Mono-S–HPLC cation-exchange column, resulting in a substance toxic to fishes by injection ($\text{LD}_{50} = 0.6 \mu\text{g}$ per 100 mg body weight). Purity of the toxic component was assessed by electrophoresis, amino-acid analysis and N-terminal amino-acid sequence determination. Proteins extracted from jellyfish tentacles were separated on a BioRad minigel apparatus, transferred to nitrocellulose membranes overlaid with the toxin-specific antibodies and detected by chemiluminescence. The western blotting kit (rabbit) was purchased from Boehringer-Mannheim. **e**, **f**, Immunofluorescent light microscopy of a resting (*e*) and discharged (*f*) nematocyst. Note that the stain revealing the toxin occurs in the tubule (*e*) and is limited to its helically arranged dots (*f*) when discharged. Bars: *e*, 2 μm ; *f*, 1 μm . For light microscopy, tentacles were sliced by a Jung CM 3000 (Leica) cryostat to sections of 8 μm , attached to glass slides with polylysine, incubated with the affinity-purified antitoxin antibody, then with goat anti-rabbit fluorescein isothiocyanate-labelled antibody (Jackson Inc.). **g**, N-terminal amino-acid sequence of the new toxin. This segment has about 60% amino-acid homology with toxic phospholipases A₂ derived from the venom of the *Heloderma* lizard and a honey bee⁵. **h**, Schematic presentation of toxin compartmentation and delivery in the nematocyst system. (1), Resting tubule, whose lumen is filled with barbs. The toxin (diamonds) is located in the folds and invaginations of the tubule's membrane (black line). (2), During discharge, toxin is translocated into the tubule while the barbs emerge and extend. (3), Toxin delivered from the everted–extended tubule through the hollow barbs.

Here, there, everywhere

Mark Ridley

Darwin's Dangerous Idea: Evolution and the Meanings of Life. By Daniel C. Dennett. Simon and Schuster. Pp. 586. \$30.

A CENTURY ago, August Weismann wrote a paper on "the all-sufficiency of natural selection". The title identifies what Daniel Dennett calls Darwin's dangerous idea: natural selection, particularly omnipotent natural selection. Evolutionary theorists would sense an ambiguity and would want to distinguish adaptation from evolution: although natural selection arguably is the universal explanation for biological adaptation, it is not for evolution. Much molecular-sequence evolution, for instance, may be by neutral drift. Dennett does not discuss neutral evolution, and his interest is clearly in design. Indeed, he is so focused on design that he almost overlooks everything else in evolution. In two chapter summaries on one page he describes "the idea that all the fruits of evolution can be explained by an algorithmic process" as "Darwin's dangerous idea" and then says that "Darwin's idea. . . provides a new explanation of the origin, by gradual accumulation, of all the design in the universe." Perhaps only adaptations are "the fruits of evolution", but the danger zone could have been more clearly defined.

(Dennett's chapter summaries contain an original device. He provides two summaries at the end of each chapter, one for the chapter just completed, the other for the chapter about to begin. They successfully add to the book's readability. The book is written for a general, educated audience, but it ranges widely and some large chunks of it are composed from Dennett's recent publications. It is always stimulating, but has some centrifugal tendency and readers will occasionally ask: "why are we going into this now?").

Dennett begins by seeking to put his finger, as exactly as possible, on what it is about natural selection that so satisfies those who like it and so offends those who do not. He has two related formulations. One is that natural selection is algorithmic. He calls a process algorithmic if it is "substrate neutral", "mindless" and produces "guaranteed results". In this sense, "every computer program is an algorithm"; Dennett distinguishes several other technical meanings of the same term.

The danger in the Darwinian algorithm is its mindlessness: algorithms reach their results even though the underlying steps are "utterly simple". Dennett contrasts the algorithmic action of natural selection with the "mind first" view, in which design in the world originates in some-

one's (usually God's) mind. He stresses how a new organ evolves from initial stages that entirely lack the interesting attributes of the final product. An eye can evolve from epidermis that has no visual sense; a wing from aerodynamically inept precursors; and — as Dennett argues, with a philosopher's care and erudition — minds, meaning, intention and morality probably evolved from earlier stages that have no features of a mind at all.

Dennett is particularly good on the recent merger of evolutionary biology and artificial intelligence. The merger is most obvious in the field of artificial life. He expounds Darwinism in a way that makes the common logic clear and he makes sense of what would otherwise be a puzzling coincidence: critics (and often the same critics) raise similar objections to both artificial intelligence and to natural selection. "No wonder, then, that there should be so much antagonism to both Darwinian thinking and Artificial Intelligence. Together they strike a fundamental blow at the last refuge to which

people have retreated in the face of the Copernican Revolution: the mind as an inner sanctum that science cannot reach."

As a relative outsider in artificial intelligence, I only wish Dennett had clarified further the extent to which the two fields stand and fall together. I can confidently identify most critics of natural selection as a bunch of twits, at least when they are writing about evolution, although I might not say so in public. With artificial intelligence there is a *prima-facie* case that some critics — Dennett discusses several, including Chomsky, Fodor and particularly Penrose — are not, and Darwinists might like to know what damage, if any, would be done to their dangerous idea if artificial intelligence succumbed to its critics. Dennett is dismissive, with what look to me like good reasons: perhaps that is why he has not thought through the Darwinian consequences of the weird counterfactual circumstance in which Penrose actually turned out to be right.

The second way Dennett describes the dangerous idea is in terms of a distinction between 'cranes' and 'skyhooks'. These are mechanical metaphors for two sorts of theory. The problem is to explain how complex designs have been built up in the world. A crane is "an honest, non-question-begging" intermediary mechanism — like natural selection, gene



KILLER instinct — female royal penguins (*Eudyptes schlegeli*) commit infanticide by removing their first egg with their feet or bill, usually on the day before the second egg is laid. From *Penguins of the World* by Pauline Reilly, an uncluttered introduction to penguin biology. Oxford University Press, £12.95 (pbk).

duplication, symbiosis, germ-line segregation, sex and learning — that helps to build physical and chemical entities into biological designs. A skyhook is an impossible mechanism by which a higher level (for example God) elevates lower levels (for example molecules) into biological or human structures.

In Dennett's view, most critics of natural selection, and probably of artificial intelligence, are hanging around for skyhooks. They cannot quite believe that algorithmic cranes do the whole job. He also uses the distinction to clarify two senses of 'reductionism'. "Good" reductionists are opposed only to skyhooks. "Greedy" reductionists try to work without cranes; they ignore intermediary theories and descend immediately to physics. Dennett has a section about Stuart Kauffman's work on order, and Kauffman seems to be a greedy reductionist who would find order in elemental mechanisms, without much need for natural selection. (He is a mild case, however. In Dennett's estimation "the world-champion greedy reductionist of all time" is B. F. Skinner.)

There is much more. There is a chapter about Stephen Jay Gould that should be widely read. It criticizes his claims — or those made on his behalf — to have killed off, or revolutionized, Darwinism and would be ideal material for graduate discussion classes. Dennett suggests (with some reasons) that Gould has skyhook-hunger, but I am unconvinced. He was moved to write the chapter because "in my own work over the years, I have often appealed to evolutionary considerations, and have almost as often run into a curious current of resistance: my appeals to Darwinian reasoning have been bluntly rejected. . . by philosophers, psychologists, linguists, anthropologists, and others who have blithely informed me that I have got my biology all wrong — I haven't been doing my homework, because Steve Gould has shown that Darwinism isn't in such good shape after all. Indeed, it is close to extinction." Dennett has enlightened things to say about the extent to which authors are responsible for their readers' misunderstandings. He also includes a wonderful list collected by Steven Pinker of the ten most amazing objections to the Darwinian explanation of language; he says they are all flourishing at the Massachusetts Institute of Technology. (They would also be a good source for entertaining exam questions.) Never in the field of scientific endeavour can so great a theory have been so misunderstood by so many with so little reason: but Dan Dennett's book is a marvellous corrective. □

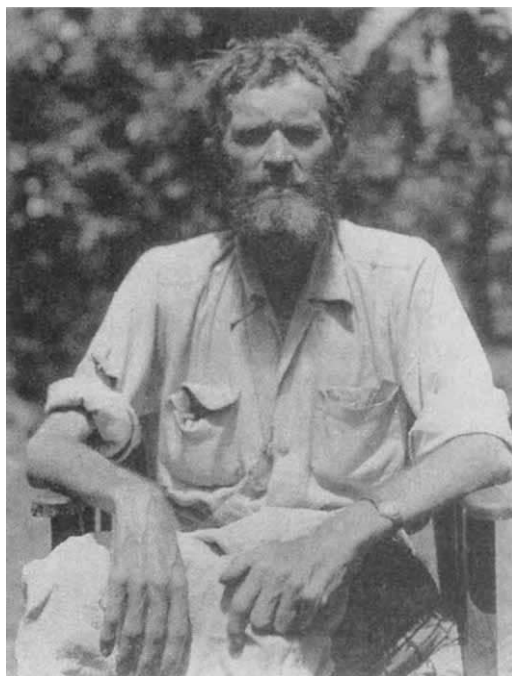
Mark Ridley is in the Departments of Anthropology and Biology, Emory University, Atlanta, Georgia 30322, USA

Anthropological imperialism

Adam Kuper

The King of the World in the Land of the Pygmies. By Joan Mark. University of Nebraska Press: 1995. Pp. 276. \$30, £28.50.

BORN into a famous Boston lineage, Patrick Putnam became a roaring boy in Harvard in the 1920s. He dabbled in anthropology, in an intellectually undistinguished department where, as Joan Mark notes, the subject "still had the aura of a profession for gentlemen, a way to



Patrick Putnam on his dude ranch, c.1952.

have foreign adventures — rather like exploring or big-game hunting but in the guise of a scholarly pursuit". Students with aspirations to do research were interrogated by Professor Tozzer, who asked them first how much money their fathers had. Putnam could give a satisfactory answer to that question at least, and he was despatched to the Congo, but he failed even by the standards of that amateur milieu. His only scientific paper was a brief note in *Man* describing a local board-game. This had been learnt during a brief visit by his stone-deaf brother, who had taught it to Patrick.

Rapidly abandoning any aspiration to do research, Putnam built what he called a "dude ranch" in the rainforest, designed to attract tourists and to provide services for journalists and scientists. The main attraction was a pygmy band that became attached to Camp Putnam. Pygmy shows were mounted for visitors and filmmakers, and Putnam tried to arrange for

some Tarzan films to be made from his camp. Although this was the era in which Western dude ranches flourished, Camp Putnam never became a viable commercial proposition, and Putnam remained a remittance-man, dependent on hand-outs from his father. He became in effect a village chief, in a raffish and brutalized colonial mode, beating his servants, shaving the hair of women who displeased him, chaining one of his African wives to a tree for several days to punish her for infidelity. A succession of three American wives were introduced into this ménage, with unhappy results, if only because he insisted on remaining polygamous.

He ran a small clinic for the government, took occasional freelance colonial jobs, and happily endorsed Belgian colonial policy: "he did not object to the pitiless exploitation of the natives," Putnam explained to the journalist Emily Hahn, "for it was a law of nature that the strong should prey on the weak; what he objected to was the hypocrisy that sometimes accompanied it, as when missionaries claimed purer motives". Some unbalanced people fed on the opportunities for petty dictatorship that European colonies offered. But like another old Congo hand, Conrad's Kurtz — a haunting parallel never mentioned by the sentimental author — such people were liable to get out of control at last; and, indeed, Putnam died in 1953, at the age of 49, barking mad, having destroyed all the furnishings of his camp and, in a final symbolic act of violence, thrown his wife's typewriter against the wall and shattered it.

Mark suggests that Putnam could never give a satisfactory account of what he knew because he could not decide "what stance he ought to take toward Africa and how he could represent what he had experienced there. . . . His was the dilemma of the self-aware white man in Africa in the twentieth century." Nothing could be more misleading. Against the grain of the author's intention, the book offers a case study of colonial pathology. Putnam was in fact a life-long dilettante, incapable of any systematic research. He belongs in the long line of colonial romantics who believed that because they could control the people around them, they understood them; but, of course, the relationship of domination precludes mutual confidence and empathetic understanding.

Mark does, however, have another story to tell that is of scientific interest. Towards the end of Putnam's life, a young Englishman, Colin Turnbull, visited his camp and was drawn into making a study of the pygmies. He produced a bestselling

From *The King of the World in the Land of the Pygmies*

book, *The Forest People*, which represented the pygmies in a classic romantic mode, as happy children of nature. (Turnbull later wrote an even more popular book on the Ik of Uganda, whom he represented in an alternative but no less romantic idiom as Hobbesian savages, stripped of culture.) Turnbull went on to study social anthropology at Oxford, and he also wrote a more sober monograph on the Mbuti, which many have treated as a reliable source on traditional pygmy life. We can now see that it was based largely on his knowledge of the Camp Putnam band, whose members had already been engaged for a generation in the tourist industry. The German authority on the pygmies, Paul Schebesta, had visited Camp Putnam and been appalled. "Nowhere else in the Ituri Forest have I found such pygmies," he wrote — and he was right. □

Adam Kuper is in the Department of Social Studies, Institute for Advanced Study, Princeton, New Jersey 08540, USA.

Chemical engines of the soul

Jim Horne

The Chemistry of Conscious States: How the Brain Changes Its Mind. By J. Allan Hobson. Little, Brown: 1995. Pp. 300. \$22.95, £15.99.

FOR Allan Hobson, dreaming embodies the innermost workings of the mind, or what he prefers to call the "brain-mind": "every detail of [a] dream can be accounted for in terms of neuronal signal-patterns". He sees no soul, or ghost in the machine; all behaviour is explicable through the activities of neurons, neurochemicals and other complex biological matter within the human brain. The mind is the brain: they are inseparable. Moreover, all mental disorders both minor and major are seen to have this biological basis. He is quite explicit: "Society readily

assumes that all these people [sufferers from mental illness] must have some history of psychological stress or trauma that has caused them to be this way. I say no." His ideal route to cure lies in correcting the brain's biology with the appropriate medication.

He postulates three cardinal brain-mind states: waking, sleeping and dreaming. The last is exemplified by his in-depth analyses of a variety of dreams, mostly from a few good raconteurs such as himself and "Delia", whose dreams often resemble psychotic states during wakefulness. Eschewing introspection on the one hand, he seems unnervingly sure about these dream interpretations and their implications.

According to Hobson, earlier proponents of the integration of brain and mind largely failed because, unlike him, they were not privy to the rapid advances in brain imaging and other sophisticated techniques. His many years of experience in psychiatry, neurology and neurophysiology have also moulded his views. But it is in the last of these fields that some may begin to see a flaw in his theory, as his work in neurophysiology is largely based on non-humans, mostly cats. A cat's brain is probably also its mind, but is Hobson extrapolating too much to us? Or does Felix indeed ponder: "*cogito, ergo sum*"?

Hobson may have the answer we have all been looking for, but his underlying evidence tends to be a bit flimsy, particularly as several basic points are oversimplified and there are sweeping generalizations. He postulates that brain-mind is largely under the control of two fundamental neurochemicals: "when we are awake our brain-minds are in the grip of one (aminergic) system, when we are dreaming our brain-minds are in the grip of a completely different (cholinergic) system", with the brainstem "a battleground for the aminergic-cholinergic system". Up to a point he may be right, but there are many other important neurochemical influences.

REM (rapid eye movement) sleep is seen to be synonymous with dreaming, and certain pronouncements about REM are, at the very least, overstatements: "REM may prevent psychosis"; "with regular exercise the aminergic system becomes more robust and is better able to control REM sleep"; and "REM is some sort of supersleep. . . one minute of REM sleep is worth five minutes of non-REM sleep". Sadly, such throwaway remarks undermine his brain-mind thesis, and at times I wondered in which of the three cardinal states some of this entertaining book was written. □

Jim Horne is at the Sleep Research Laboratory, University of Loughborough, Leicestershire LE11 3TU, UK.



ANCIENT Mayan incensarios such as this were often destroyed as heathen idols by the sixteenth-century Spanish conquerors of the New World. This picture is taken from the fifth edition of the comprehensive and lavishly illustrated *The Ancient Maya* by Robert J. Sharer. Stanford University Press, £74, £55 (hbk), \$24.95, £17.95 (pbk).

Here are only numbers ratified

David M. Raup

Extinction Rates. Edited by John H. Lawton and Robert M. May. Oxford University Press: 1995. Pp. 233. £17.95, \$27.95 (pbk).

WHAT do we really know about present-day rates of species extinction? Can we convert current estimates into rigorous assessments of probable loss of biodiversity in the future? It is indeed difficult for the general reader (scientist or not) to



find satisfying answers. Most of the summary literature and press coverage is emotionally and politically charged, and rarely delves more deeply than to cite a few dramatic anecdotes to support an advocacy position, for or against. In different treatments we read statements with wildly different implications. In one version, the fact that most native Hawaiian bird species are now extinct is extrapolated to global apocalypse. But then we read (much less often) that despite 98 per cent deforestation in the eastern United States in the seventeenth and eighteenth centuries, almost no species extinctions can be documented. Against this backdrop, *Extinction Rates* is a breath of fresh air.

Lawton and May have assembled a well integrated set of 14 research articles and reviews that attempt to put the study of extinction rates on a more rigorous footing. Although the project started out as a series of presentations at a Royal Society discussion meeting held in London in October 1993, the papers have been extensively revised by their authors and new material has been added to

make a coherent whole. The result is an excellent overview of what we do and don't know about the biodiversity problem. The first chapter (by May, Lawton and N. Stork) is especially good as a general statement and it also serves well as an abstract of the volume; in 20 pages, the reader is introduced to the extinction problem, warts and all, and is given a summary of the main points to follow. The basic thesis throughout is that conservation biologists can (and should) move beyond the trading of anecdotes and beyond the conventional estimates of rates derived from species-area relationships and habitat loss. Over and over again, the plea is: "Let's go back to hard science and do it right!"

Historical data on species extinctions play an important role in the book, from D. Jablonski's concise summary of the rich palaeontological record of the past 600 million years, through J. B. C. Jackson's analysis of tropical marine communities and G. R. Coope's evaluation of insect extinctions (or lack thereof) during the most recent ice age, to several historical studies of the effects of human habitation on extinction rates (for example S. L. Pimm, M. P. Moulton and L. J. Justice on bird extinctions on Pacific Islands and W. Greuter on higher plants in the Mediterranean region). Each of these chapters has some surprises. Both Jackson and Coope emphasize the relatively low rates of species loss during the Pleistocene glacial periods, despite repeated losses of habitats. For the marine biota, Jackson's interpretation is that although the onset of glaciation did cause a significant pulse of extinction, the equally severe stresses during the long sequence of glacial and interglacial periods did not, because species susceptible to these stresses had already been eliminated. This idea of filtering out susceptible species by early stresses is echoed elsewhere in the volume at different scales, with Pimm and colleagues showing that the number of currently endangered bird species on Pacific Islands varies inversely with the antiquity of human occupation. And Greuter notes that current rates of loss of plant species are low where human habitation is ancient (as in the Mediterranean) and high where colonization was more recent (as in south-western Australia).

Most of the later chapters are devoted to attempts to find new approaches to estimating extinction rates, for both the past and the future. What makes one species or group more or less at risk of extinction? This is a complex and difficult question, and except for the widely observed fact that geographically restricted organisms are more prone to extinction than cosmopolitan ones, there are few clear-cut correlates with extinction risk. Nevertheless, several of the contributions, notably Lawton's superb treat-

ment of population dynamics and extinction, are constructive. There is also a valiant attempt by G. M. Mace to use published lists of threatened species (such as *The Red Lists* and *Red Data Books* of the International Union for the Conservation of Nature and Natural Resources) to make rigorous estimates of present and future extinction rates. Of much larger scope is S. Nee and colleagues' innovative use of molecular phylogenies of evolutionary groups (clades) to infer the risk of total extinction of such groups.

Taken as a whole, *Extinction Rates* is a gold mine of hard data and solid science that should be read by all with a serious interest in biodiversity — past, present and future. □

David M. Raup is emeritus professor at the University of Chicago, Chicago, Illinois 60637, USA.

New in paperback

Trees and Woodland in the British Landscape by Oliver Rackham. Weidenfeld and Nicolson, £12.99. A revised version of this authoritative history of Britain's trees, woods and hedgerows, first published in 1976.

The Biophilia Hypothesis edited by Stephen R. Kellert and Edward O. Wilson. Island, \$17.95. Wide-ranging explorations of Wilson's influential idea that humans have an "innate affinity for all living things". Contributors include Wilson ("Biophilia and the Conservation Ethic"), Jared Diamond ("New Guineans and Their Natural World") and Dorion Sagan and Lynn Margulis ("God, Gaia, and Biophilia").

Adaptation and Environment by Robert N. Brandon. Princeton University Press, \$16.95, £14.50. "Brandon aims to give an accurate account of natural selection and the concept of environment needed by that theory. He tackles the issue of tautology and the nature of adaptationist explanation, as well as the unit of selection. The book is written for philosophers, and professionals will need to know what is in it," wrote Mark Ridley in a review in *Nature* 347, 345 (1990).

Crisis in the World's Fisheries: People, Problems, and Politics by James R. McGoodwin. Stanford University Press, £10.95 (distributed in the UK by CUP). Aims to provide a broad overview and fundamental reassessment of fisheries management policies around the world.

Out of Control: The New Biology of Machines, Social Systems, and the Economic World by Kevin Kelly. Addison-Wesley, \$16. The author, a self-confessed "techno-transcendentalist", chronicles the "dawn of a new era in which the machines and systems... are so complex and autonomous as to be indistinguishable from living things".

Bacteriorhodopsin as a model for proton pumps

Janos K. Lanyi

According to a long-standing hypothesis, membrane pumps function by flip-flopping between two protein conformations that allow alternative access of the ion binding site to the two membrane surfaces. Site-specific mutagenesis, time-resolved spectroscopy and X-ray diffraction confirm this mechanism for bacteriorhodopsin, and implicate change of electrostatic interaction at the active site as the trigger for the global protein conformation change during the proton transport cycle.

THE interiors of cells and organelles are kept at compositions different from their exterior by active transport of ions across cellular and intracellular membranes. Such transport requires that a spontaneous reaction at the active site of a membrane enzyme be coupled to the unidirectional movement of an ion across the membranes so as to cause uptake at one surface and release at the other, even in the face of an opposing transmembrane electrochemical potential. In some proton pumps based on redox reactions, quinones act as mobile carriers for electrons and hydrogen within the membrane phase¹, but in others a proton wire² or alternating protein conformations³⁻⁷ have been suggested. In the last mechanism, the chemical changes at the active site (the driving reaction) alter the conformation of the protein so as to regulate the conduction of the transported ion between its binding site and the two membrane surfaces. Such a relationship of the active site, the protein conformation, and the proton conduction pathways has been described so far only in bacteriorhodopsin.

Bacteriorhodopsin

The light-driven proton pump of halobacteria^{8,9} consists of seven transmembrane helices that enclose a cavity containing a retinal bound to the ϵ -amino group of Lys 216 through a protonated Schiff base (Fig. 1a). The structure is described by an atomic model based on a 3.5 Å resolution map from electron cryo-microscopy¹⁰. The trajectory of the transported proton is defined by the Schiff base and two aspartate residues, Asp 85 and Asp 96. After photoisomerization of the retinal from all-*trans* to 13-*cis* the Schiff base donates a proton to the anionic Asp 85, followed by its reprotonation from the initially protonated Asp 96. These internal proton transfers cause proton release at the extracellular surface and proton uptake at the cytoplasmic surface, and thereby result in the net translocation of a proton.

Many investigators have anticipated that if there are fundamental and common principles for ion pumps, the study of this small (M_r , 26K) protein would reveal them. Indeed, kinetic models and analyses of energy flow during the photocycle demonstrated¹¹⁻¹³ that, although initiated by photoexcitation, the ensuing thermal steps of the transport cycle are much like those in any complex enzyme reaction. Thus, although the proton transfers inside the protein take place near equilibrium, the proton release and uptake at the protein surfaces occur with transiently lowered and elevated pK_a values¹⁴⁻¹⁶, respectively; that is, the proton electrochemical potential is created as the excess free energy acquired by the retinal chromophore is dissipated by protein residues. Also in analogy with enzymes, the protein conformation undergoes distinct changes during the reaction sequence¹⁷⁻¹⁹. The reaction pathway is illustrated in Fig. 2.

The consequences of replacing various residues have been previously explored by kinetic spectroscopy in the visible and the infrared. Much of this work was directed towards identifying the path of the transported proton and describing the kinetics

of the reaction cycle (the 'photocycle'). The switch in the connectivity of the Schiff base from Asp 85 to Asp 96 was recognized as the equivalent of the alternating access in other pumps¹¹, and identified as a unidirectional step between the deprotonation and the subsequent reprotonation of the Schiff base¹³. More recent studies have been on the origins of the protein conformation change in the photocycle, and the way it is linked to this reprotonation switch. The properties of a group of mutated proteins with the proton acceptor Asp 85 replaced suggested that coulombic interaction between the positively charged proton donor and the negatively charged proton acceptor at the active site regulates the global protein conformation²⁰. The unphotolysed state of the protein is stabilized by the binding energy of a complex formed by the Schiff base, Asp 85, a few other charged residues, and liganded water. In this state (conformation E) the Schiff base proton is connected to Asp 85, and thus the extracellular side. Photoisomerization of the retinal²¹ displaces the Schiff base relative to Asp 85, and the new geometry, as well as bond torsions along the retinal chain immobilized by residues in the binding pocket, favours transfer of the Schiff base proton to the aspartate. Once the Schiff base/Asp 85 ion pair is thereby converted to a neutral pair, the protein relaxes into its default conformation (conformation C). Difference projection maps from X-ray and electron diffraction indicate that the conformation change at this time in the cycle is mostly a tilt of helix F at the cytoplasmic surface away from the centre of the seven-helical structure, and reorganization of the cytoplasmic side of helix G¹⁷⁻¹⁹. In conformation C the Schiff base is connected to the cytoplasmic side²⁰, and its access to Asp 85, and therefore to the extracellular side, is broken. The electrostatic nature of the trigger for the transition from conformation E to C is demonstrated by the finding that it occurs not only in the photocycle of the wild-type protein but also in the unilluminated D85N mutant when the pH is raised to deprotonate the Schiff base²⁰.

Proton transport in bacteriorhodopsin is therefore accomplished according to the alternating access model of ion pumps. Unexpectedly, the initial state of the polypeptide chain is an inherently high-energy conformation, and it is the release of this conformational energy upon the initial proton transfer from Schiff base to Asp 85 that provides for the reprotonation switch. This means that at the end of the photocycle, part of the excess free energy acquired will be retained in the protein. Such a thermodynamic cycle is not unlike what was recently suggested for myosin-actin interaction on ATP binding and hydrolysis²².

It appears that in bacteriorhodopsin the protein conformation alternates between two states according to the presence or absence of a local interaction at the active site. Is this a general principle? Certainly, in this respect, bacteriorhodopsin is similar to visual and sensory rhodopsins. The former initiates the cascade of visual transduction when its Schiff base is deprotonated in the meta II photointermediate, and thereupon binds transducin. Replacement of the anionic Glu 113 (the equivalent of

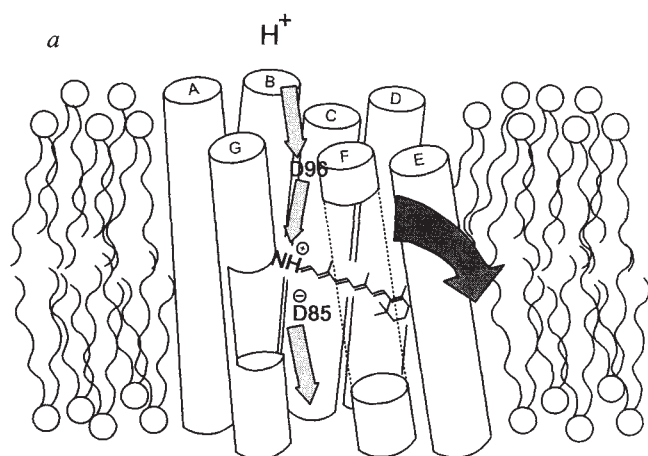


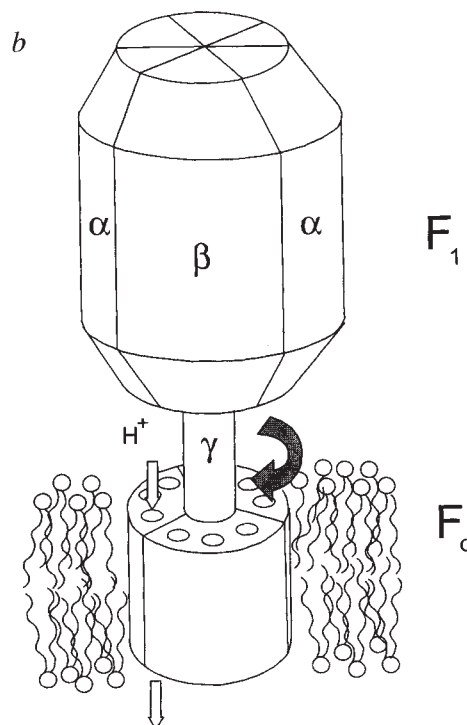
FIG. 1 Schematic drawings of bacteriorhodopsin (a) and the F_0F_1 -ATPase (b). The pathways of protons are indicated by straight arrows, the proposed tilt of helix F and rotation of the γ -subunit by curved arrows.

Asp 85 in bacteriorhodopsin) with glutamine, or the positively charged Lys 296 (the equivalent to Lys 216 in bacteriorhodopsin, that is, the Schiff base) with glycine, resulted in constitutive binding of transducin²³. The contribution of electrostatics at the active site to the stability of the overall structure is emphasized by the finding that when the coulombic interaction was made weaker by moving the anion one helical turn away from the Schiff base, photoisomerization of the retinal (from 11-*cis* to all-*trans* in vertebrate rhodopsins) caused transducin binding even without deprotonation of the Schiff base²⁴. Remarkably, the sensory rhodopsin I of the halobacteria that normally functions as a light-receptor with no transport function, uses an aspartate near the retinal to transport protons, like bacteriorhodopsin, in the absence of its transducer protein²⁵. Because the rhodopsins seem to be prototypes for biogenic amine-binding receptors, or perhaps even for the entire G-protein linked receptor superfamily^{26,27}, this kind of behaviour might represent a general mechanism for conformational control.

In fact, the idea of active-site stabilization of protein conformation is implicit in models proposed for a wide variety of ionic pumps. This is illustrated in two pumps of greater complexity than bacteriorhodopsin.

Cytochrome c oxidase and F_0F_1 -ATPase

Cytochrome c oxidase²⁸⁻³² is a representative of a large group of terminal oxidases of the respiratory chain. In these enzymes four electrons are used sequentially to reduce O_2 . Four protons react with the dioxygen to form water, and another four are translocated across the membrane³. The structure is known to about 15 Å resolution³³. It is large and complex in eukaryotes (M_r about 200K, 13 subunits), but simpler in prokaryotes which contain only subunits I-III (ref. 34). Subunits I and II are sufficient for both oxygen reduction and proton transport. Subunit II contains Cu_A , the entry point for electrons from cytochrome c. Subunit I contains 12 putative transmembrane helices, labelled I to XII, and two metal centres: haem *a* and haem a_3 , the Cu_B binuclear centre. The metal centres are located near the cytoplasmic side, and oriented so that the path of the electrons is roughly parallel to the plane of the membrane. The transport of protons occurs in two steps after the binuclear centre is partially reduced and dioxygen is bound to haem a_3 . Many mutations along the hydrophilic surface of helix VIII abolish oxygen reduction and transport but do not reveal which groups carry the protons to and from the active site, and bind protons at the binuclear centre³¹. Replacement of Asp 134 located in the loop between the matrix ends of helices II and III abolishes transport



but not the redox reaction³⁵. Thus, it is not yet clear how the electron transfer steps and the proton transfer steps are coupled, and whether this coupling is direct or indirect. Most proposed models of proton translocation are based on shuttling of ligands to and from haem a_3 or Cu_B . In a proposed direct coupling model³⁶, for example, His 284 coordinated to Cu_B plays the role of such a ligand, carrying 2×2 protons per cycle across the internal barrier.

Schemes that account for the proton transport in terms of alternating input and output states in the reaction cycle make use of many observations that the protein can assume two conformations³⁷. Although direct evidence for protein conformation changes during the reaction cycle is lacking, the kinetics indicate³⁸ that as the enzyme undergoes two cycles of electron transfer there must be an input conformation that allows the reduction of haem *a* and the uptake of two protons from the matrix side, and an output conformation that allows the release of two protons to the cytoplasmic side at the binuclear centre.

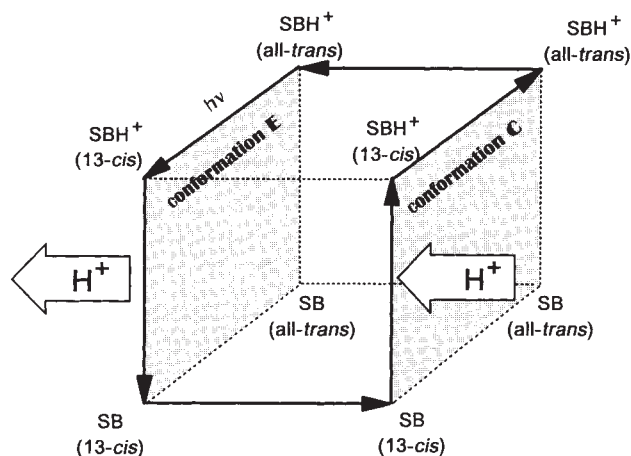


FIG. 2 The bacteriorhodopsin photoreaction cycle, drawn similarly to the 'cubic scheme' for cytochrome c oxidase^{3,30}. The retinal is either all-*trans* or 13-*cis*, the Schiff base protonated (SBH^+) or unprotonated (SB), and the protein conformation is either E (with extracellular access of the Schiff base proton) or C (with cytoplasmic access).

The F_0F_1 -ATPase of the mitochondrial membrane represents a group of proteins with very complex architecture that can function either as ATP synthases driven by proton-motive force or proton pumps driven by ATP hydrolysis^{39–42}. The total M_r is ~500K. The dissociable, water-soluble, bulb-shaped F_1 complex contains subunits α , β , γ , δ and ϵ (with a stoichiometry 3:3:1:1:1), whereas the membrane-immersed F_0 complex contains numerous kinds of small hydrophobic peptides, in particular an estimated 9–12 copies of the c subunit (Fig. 1b). The structure of most of F_1 is now known to 2.8 Å resolution⁴². In the pump mode the single reaction cycle consists of the hydrolysis of 3 ATP molecules by F_1 , and the translocation of 9–12 protons by F_0 . A great deal of kinetic, and now also structural, evidence indicates the existence of three interacting hydrolytic sites, one on each β -subunit, and the sequential changes of these sites as they progress through the non-binding, the ATP-binding and the ADP + P_i -binding states, each 120° out of phase with the others^{41–44}. The F_0 complex has been visualized as a rotor assembly of subunits, with 9–12 stops that allow an interrupted flow of protons⁴⁵. The transient binding of these protons may be to Asp 61 of each c subunit⁴⁰. The separation of the functions of the F_1 and F_0 complexes is suggested by the existence of an analogous but Na^+ -translocating ATPase⁴⁶, and the fact that a hybrid protein containing F_1 from the proton pump and F_0 from the sodium pump transports Na^+ (ref. 47). Transmission of free energy between F_1 and F_0 is through a slender stalk that connects them, by what appears to be rotation of the γ -subunit to connect, one by one, with the β -subunits according to whether their hydrolytic sites are empty or contain ATP, or ADP + P_i ^{48,49}. This long-range conformational change would allow the directional transfer of three or four protons across the membrane for each third of the cycle.

Discussion

The common element in these three pumps is that the measured or postulated alternative protein conformations are regulated by interaction of the active site with the rest of the protein. The results with bacteriorhodopsin suggest that it is feasible for local changes at the active site upon energization to affect the overall protein structure. Thus, the group transfer energy of the chemical reaction (ATP hydrolysis, electron and/or proton transfer and so on) may be sufficient to shift one structural alternative to the other. In the simpler pumps the excess free energy is either generated at the binding site (in bacteriorhodopsin), or transferred directly to the ion binding site (in cytochrome c oxidase). In more complex pumps the transmission of free energy

from the site(s) of the driving reaction to the site of ion translocation must be through structures that offer mechanical coupling of conformation changes. When the number of ions transported per cycle is large the transducer appears to be a rotor, with multiple binding sites, that turns to expose one site at a time to the membrane surfaces (in the F_0F_1 -ATPase). Thus, at one extreme, ion pumps appear to be based on principles akin to muscle contraction, but at the other they resemble molecular motors otherwise used for locomotion, as in bacterial flagella.

In evaluating the generality of this hypothesis, we will need to know more about the nature and energetics of the protein conformational changes. Efforts are underway to describe the conformational change in bacteriorhodopsin at a higher resolution and in three dimensions, and to decide whether it consists of flip-flopping between two alternatives or of a series of conformational shifts that originate at the active site and spread progressively over the protein, as suggested in a different context for myoglobin⁵⁰. In the F_0F_1 -ATPase the structural differences among the three α/β subunits, and their connection to the γ -subunit, are well documented⁴⁰, and further work will reveal how these are linked to the states of the nucleotide binding sites and the position of the proposed rotor of the F_0 complex. In cytochrome oxidase there is only indirect evidence for an important conformational change.

The second question concerns the energetics of the alternating conformations. The results with bacteriorhodopsin suggest that the connection between the active site and the protein conformation might be primarily electrostatic. Both the redox states of the iron and copper centres and the protonation states of the proton transfer groups in cytochrome c oxidase, for example, in the histidine cycle³⁶, and the binding, hydrolysis, and release of ATP and ADP in the F_0F_1 -ATPase⁴¹ will be affected by the net charge and the geometry of the charges at the active site.

Despite the many uncertainties discussed above, it appears that a common mechanism for energy coupling in proton pumps (and signal transduction in receptors) is beginning to emerge. Results with bacteriorhodopsin, the simplest of the pumps, confirm the long-held alternating access hypothesis of active ion translocation, and attribute the conformational transition of the protein to changes in electrostatic interactions at the active site. The continued elucidation of these principles, as well as the mechanistic details unique to each protein, should provide a much improved understanding of ion pumps within the next few years. □

Janos K. Lanyi is at the Department of Physiology and Biophysics, University of California, Irvine, California 92717, USA.

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ACKNOWLEDGEMENTS. The author's research is supported by the NIH, DOE and NSF. He thanks R. G. Gennis, R. Henderson, B. Malmström, T. Poulis, B. Rosen, J. E. Walker, S. H. White and M. Wikström for valuable suggestions.

Episodic ejection of relativistic jets by the X-ray transient GRO J1655 – 40

R. M. Hjellming & M. P. Rupen

National Radio Astronomy Observatory, Socorro, New Mexico 87801-0379, USA

GRO J1655 – 40, a recently discovered black-hole candidate, is an ideal system for studying jets of material from the accretion disk around a black hole. Observations of the radio emission show two highly collimated relativistic jets, one on each side of the source, which expand and decay over a few days. The jet ejection, at 92% of the speed of light, appears episodic and asymmetric; the alternate brightening and fading of the jets cannot be explained by relativistic beaming.

COLLIMATED jets are a common astrophysical phenomenon; they are thought to be associated with neutron stars, stellar-mass black holes, and the massive black holes which may power active galactic nuclei (AGN) and quasars^{1,2}. The angular momentum of the gas being accreted by these objects forces the gas into a disk-like structure. The rotation axis of this accretion disk provides a natural preferred direction for the jets, while the energy for both the jets and the high-energy radiation comes from the gravitational energy released as the gas falls towards the compact object. Exactly how the jets are created is still largely unknown, however, in part because these objects are difficult to study in detail. Quasars and AGN generally lie at large distances, restricting the sizes of the structures that can be resolved. In addition, the large physical size of these objects means that it takes years to see small changes in the jets. Ideally, one would like to study sources that have the same relativistic jets and high-energy radiation as the quasars and AGN, yet are near enough and bright enough for small-scale features to be resolved, and evolve quickly enough for changes in source activity and structure to be monitored.

The Galactic black-hole candidate GRO J1655 – 40 seems an ideal source for such a study. It was first detected as an X-ray source³ by the Burst and Transient Source Experiment (BATSE) on the Compton Gamma-Ray Observatory on 27 July 1994 (day 9560, where we define day number as Julian day (JD) minus 2440000.5), and as a radio source⁴ by the Molonglo Synthesis Array on day 9570. The jets show apparent superluminal motion^{5,6}, implying that the radio-emitting plasma is moving at speeds close to that of light. It lies at a distance of about 3 kpc (refs 3, 5, 17) from Earth, and the optical counterpart has recently been identified⁷. The motion of the jet material is so rapid that distinct motions can be seen from one hour to the next.

We have used the Very Large Array (VLA) to follow the evolution of the radio jets between days 9576 and 9708, and the Very Long Baseline Array (VLBA) to study in detail the ejection of material between days 9583 and 9616. We find two oppositely directed jets, moving almost perpendicular (85°) to the line of sight. The relative brightness of the two jets varies, to the extent that either of the two may be dominant, depending on the observing date. The bright parts of the jets fade over periods ranging from a few days to two months. Brightness asymmetries in other sources have been interpreted as relativistic beaming, in which material moving very rapidly towards us appears brighter than that moving very rapidly away; however, both jets of GRO J1655 – 40 lie almost in the plane of the sky, so beaming cannot explain the observed brightness ratios. Although the jets are nearly straight, some emission does appear displaced from the jet axis. This might arise either from structure within the jets, or from rotation of the jets about the jet axis. If the latter

is correct, this could be an indication either of the orbital period of the binary, or of the precession period of a warped accretion disk.

GRO J1655 – 40 both confirms the existing general picture^{1,2} of astrophysical jets, and illustrates how little they are understood. The association of the high-energy photons seen by the Gamma-Ray Observatory with the high-energy mass motions seen by the VLBA is predicted by the standard model, in which both originate in an accretion disk around a black hole; however, the detailed physics behind this association remains obscure. Nor can current models reproduce the observed brightness asymmetries, or explain the most obvious properties (such as decay rates and ejection histories) of the jets themselves. The changes and complexity seen here may ultimately provide new clues into how relativistic jets arise and evolve.

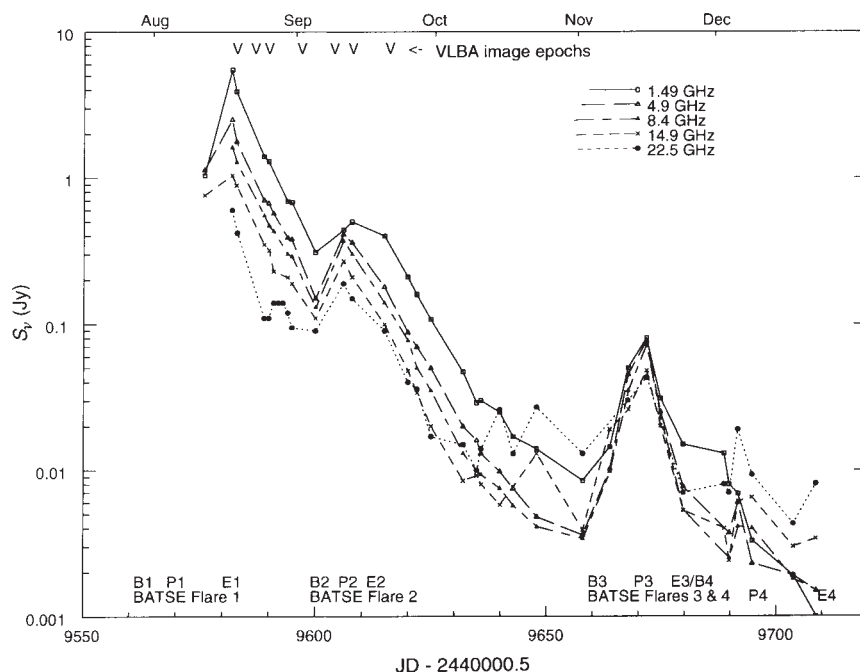
VLA radio lightcurves and images

The radio lightcurves of GRO J1655 – 40, as determined from the VLA observations, are displayed in Fig. 1. The lightcurves are dominated by three radio flares, becoming progressively weaker in time; the first two of these share an *e*-folding decay time of about six days, whereas the third shows a sharper decline. Although the source was initially (day 9576) optically thick, by day 9582 the spectral index had reached an optically-thin state with $\alpha \approx -0.65$ (where the flux density S_ν is proportional to ν^α for frequency ν), a value which remained nearly constant through most of the subsequent evolution. This, combined with the time-variability and high brightness temperatures ($\geq 10^8$ K), implies that the radio emission is predominantly synchrotron, from relativistic electrons interacting with magnetic fields.

The first VLA observation (day 9576), with antennas in the 10-km configuration, showed an unresolved source (<100 mas); by the time of the radio peak six days later, the source had expanded enough to be resolved at 22 GHz, with an east–west size of 200 mas. A selection of subsequent VLA images, beginning the following day, is shown in Fig. 2. The source appears to be made up of a number of discrete, fast-moving components ejected at a roughly constant position angle from a central source; although these ejecta gradually die out as they move away from the core, the core itself is always present as the brightest part of the image. Although the initial ejection is to the northeast (NE), with only marginal signs of a corresponding knot to the southwest (SW), some of the subsequent ejecta (see, for instance, day 9606) are stronger to the SW.

Figure 3 summarizes the proper motions of the ejecta as derived from the complete set of 22 epochs of VLA images, spanning days 9582 to 9647. Comparison with the VLBA images (discussed below) shows that spatial averaging of underlying structures is important, resulting in an apparent deceleration as the outermost ejecta fade in strength. These and other data are

FIG. 1 Radio lightcurves for GRO J1655–40, plotted as a function of JD–2440000.5 (day number), for frequencies (GHz) of 1.49 (squares, solid line), 4.9 (open triangles, long-dashed line), 8.4 (filled triangles, short/long-dashed line), 14.9 (crosses, short-dashed line) and 22.5 (filled circles, dotted line). The Vs at the top of the figure identify the epochs of the VLBA images; at the bottom of the figure, the B, P and E identify the beginning, peak and end of X-ray flares (as determined by BATSE³) which we identify as numbers, 1, 2, 3 and 4. The peak observed flux density was 5.5 Jy at 1.49 GHz on day 9582.



consistent with constant intrinsic proper motions of 54 mas d^{-1} to the NE and 45 mas day^{-1} to the SW, corresponding to three major ejection events on days 9577.5, 9584 and 9597.

The VLA data show a central radio source in all 38 epochs, with mean absolute position (J2000) of right ascension $16 \text{ h } 54 \text{ min } 0.156 \text{ s}$, declination $-39^\circ 50' 44.7''$, with estimated errors of 0.02 s and $1''$, respectively. This is consistent with the position for the optical outburst (Bailyn *et al.*⁷). Whether the central source is physically distinct from the jets, or consists of unresolved, young, jet material, is not clear. However, the radio lightcurves sometimes show an excess at the highest frequencies, particularly obvious after day 9620 in Fig. 1, and both the intensity level and the rapid variation of that excess are consistent with the late-time, slowly decaying emission seen in other X-ray sources, such as GS2023+338 (ref. 8) and GRO J0442+32 (ref. 9). Unfortunately the VLA and VLBA data discussed in this Article do not have the resolution to distinguish a core by its flat spectral index.

Both the ejecta which separate from the central source, and the central source itself, share in the overall decay seen in the radio lightcurves, with the radio peaks corresponding to major ejections. However, the individual ejecta, as seen in the VLA images, can have very different lifetimes. In Fig. 3 we see the NE ejection between days 9577 and 9588 has faded entirely by day 9602, whereas that from day 9597 can be followed for at least 50 days. As the underlying proper motions appear constant, the fact that one sees emission arcseconds from the centre shows that these components can survive for a much longer time than the average decay e -folding time of six days.

VLBA images of rapidly moving twin jets

Figure 4 shows the highest-resolution image sequence ever made during flaring of an X-ray transient in our Galaxy. These images were made from seven six-hour VLBA observations at a frequency of 1.6 GHz , with the first taking place on day 9853 (just after the first radio peak), and the last on day 9616 (roughly a week after the second radio peak). These data are both more complicated and more difficult to analyse than the VLA data, for three reasons. First, the lack of a nearby very-long-baseline interferometry (VLBI) calibrator made phase self-calibration a necessity, eliminating all absolute positional information, and leaving the alignment of the different images a free parameter. We assume that the brightest point in each image is the (station-

ary) centre of ejection, partly because this seems physically reasonable, but primarily because the proper motions and (in those cases for which we have simultaneous measurements) the component positions thus derived agree with those measured in the VLA images. Second, the VLBA data are not sensitive to structure on large angular scales, owing to Fourier filtering by an interferometer with a shortest projected spacing of 40 km , corresponding to about 1 arcsec at 1.6 GHz . This is reflected in the discrepancy between the VLA and VLBA flux densities for the two days for which we have simultaneous measurements: the VLA measurements indicate flux densities at 1.6 GHz of 3.4 Jy and 1.23 Jy on days 9583 and 9590 respectively, whereas the VLBA images presented here recovered only 3.2 and 1.1 Jy . Amplitude self-calibration did not improve the results for any of the seven epochs, reflecting the excellent amplitude calibration of the VLBA antennas. Last, the apparent proper motion of approximately 50 mas d^{-1} indicated by the VLA images, and supported by the VLBA data, corresponds to about 14 mas proper motion over the course of each six-hour VLBA observation; for this reason the images were all made with beam-widths larger than 14 mas .

The evolving structures shown in Fig. 4 are highly elongated and roughly linear, with rapidly changing twin-jet structures near the centre of ejection. As the ejecta move out from the centre they both decline in flux and expand perpendicular to the direction of motion. The flux decay rate varies along the jet; the strong component in the middle of the NE ejecta, which dominates those ejecta on day 9583, fades to a weak presence by day 9587, and nearly disappears by day 9590. At the same time the more extended structure both expands transversely and decays, leading to a relative weakening of the outer (older) portions compared to the inner (younger) ones.

Tingay *et al.*⁵ and Murphy *et al.*⁶ have made a series of VLBI images of GRO J1655–40 based on daily observations with the Southern Hemisphere VLBI Experiment (SHEVE) array, covering days 9586–89, roughly overlapping with our second and third VLBA epochs. The VLBA and SHEVE images are consistent in the major structures, and the SHEVE proper motion of $65 \pm 5 \text{ mas d}^{-1}$ is consistent with the 62 mas d^{-1} motion of the outer edge of the early NE ejecta. One possible discrepancy is that their maps show a one-sided jet to the NE, whereas ours show some significant flux 200 mas SW of the bright core; this is probably not significant as their $123 \times 26 \text{ mas}$

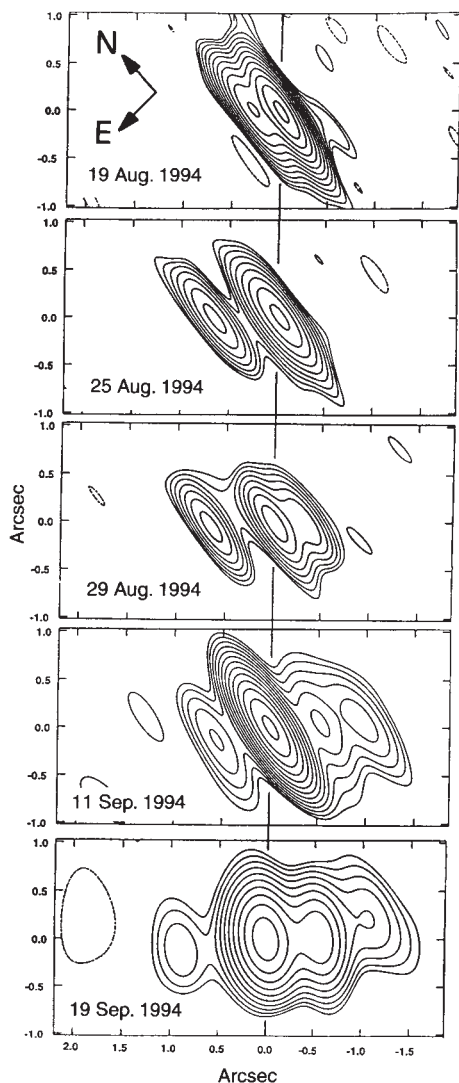


FIG. 2 Five images of GRO J1655–40 made from self-calibrated 22-GHz VLA data taken on days 9583 (top panel), 9589, 9593, 9606 and 9614 (bottom panel). All have been rotated anticlockwise by 43° . The east–west half-power beam-widths are 0.20, 0.23, 0.22, 0.2 and 0.4 arcsec, respectively. The vertical line indicates the adopted position for the stationary core, as discussed in the text. Contouring is $1.414^n \times S_0$, where $S_0 = 4, 4, 2, 5$ and 1 mJy per beam, and the peak flux densities are 280, 69, 28, 34 and 28 mJy per beam, respectively.

beam is elongated only 30° from the jet axis. As mentioned above, the VLBI images of neither group have absolute positional information, and this, combined with the complexity of the source structure and calibration uncertainties, leaves ambiguities in the mapping. Because of somewhat better Fourier sampling, the superior *a priori* amplitude calibration of the VLBA antennas, and consistency with the VLA images, we believe that the two-sided jet structure shown in Fig. 4 is real.

Kinematic model

The simplest model one can attempt to fit to the images in Fig. 4 is twin jet ejection at an angle with respect to the observer which remains constant in time. This model has five parameters: the jet velocity (v), the distance (d), identification of one side as approaching and the other receding, the inclination angle with respect to the line of sight (i), and the apparent position angle on the sky. If one identifies the NE and SW ejecta with proper motions of $\mu_- = 54$ and $\mu_+ = 45$ mas d^{-1} , respectively, along a position angle of 47° , and attributes the difference as due to

relativistic time delay, then the proper motion equation, $\mu_{\pm} = (v/d) \sin i / [1 \pm (v/c) \cos i]$ where c is the velocity of light, yields limits on both the inclination and the velocity: $i \leq 84.8^\circ$, $(v/d) \geq 49$ mas d^{-1} . For a distance of 3.2 kpc, this corresponds to $v \geq 0.91c$, implying $84.3^\circ \leq i \leq 84.8^\circ$.

In the simple linear ejection model, the positions of the flux-density maxima must be ascribed to the vagaries of the source of the jets. But the maxima seem to occur at regular intervals, and the jets “wobble” slightly about the best-fit position angle. These fluctuations occur on spatial scales of approximately 150 mas, corresponding to a time span of about three days for proper motions of ~ 50 mas d^{-1} . This behaviour is physically reasonable, as shown by the case SS433 (refs 11–15), which shows periodic changes of the radio-jet axis as well as periodic red- and blue-shifts of optical and X-ray emission lines. Simulations of our VLBA observations show that even jets moving as rapidly as these cannot produce the “wiggly” structures seen in our images. Assuming that the wiggles are due to kinematics, we were able to fit them to an SS433-like kinematic model. For this fit the jets are rotating clockwise about the jet axis with a period P of 3 ± 0.2 d, the angle between the jets and the jet axis is $\psi = 2^\circ \pm 0.5^\circ$, and the jet axis is inclined to the line of sight at an angle $i = 85^\circ \pm 2^\circ$, with a position angle of $47^\circ \pm 1^\circ$. The jets move at $v = (0.92 \pm 0.02)c$, with the NE jet approaching and the SW receding. This geometry is sketched in Fig. 5.

Contrary to the usual model for extragalactic jets, and for the other highly relativistic Galactic jet source GRS1915+105 (ref. 16), the difference in strength between the emission on either side of the centre in GRO J1655–40 cannot be ascribed to relativistic Doppler boosting. A velocity of $0.92c$ gives a Doppler factor $\gamma \equiv [1 - (v/c)^2]^{-1/2} = 2.55$, but as the inclination to the line of sight is so large, the resulting Doppler boost is at most 60–90%. The observed asymmetry in brightness is often much higher, and in any case flips from side to side. A model in which the NE and SW jets swap roles as approaching and receding is untenable, as the ejecta are so nearly collinear. The jets themselves must be intrinsically asymmetric, and the sense of that

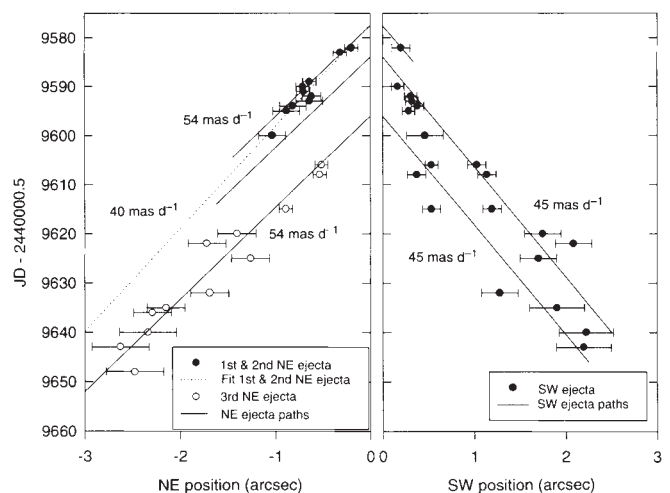


FIG. 3 Proper motion of identifiable components in 22-GHz VLA images, for days 9581 to 9647, plotted as a function of JD – 2440000.5 (day number). All components shown here were ejected during the first radio flare. The abscissa is the distance from the central source along a position angle of 47° . In the left panel the first and second sets of NE ejecta are plotted with filled circles, with full and dotted lines showing how the initial apparent proper motion of 54 mas d^{-1} seems to change to an average of 40 mas d^{-1} ; the proper motions of the third set of NE ejecta are plotted with open circles, together with a fitted line with slope 54 mas d^{-1} . The right panel displays a similar plot for the SW ejecta. The solid lines, of slope 54 and 45 mas d^{-1} for the NE and SW ejecta respectively, indicate our best fits to a combination of VLA and VLBA data (see text).

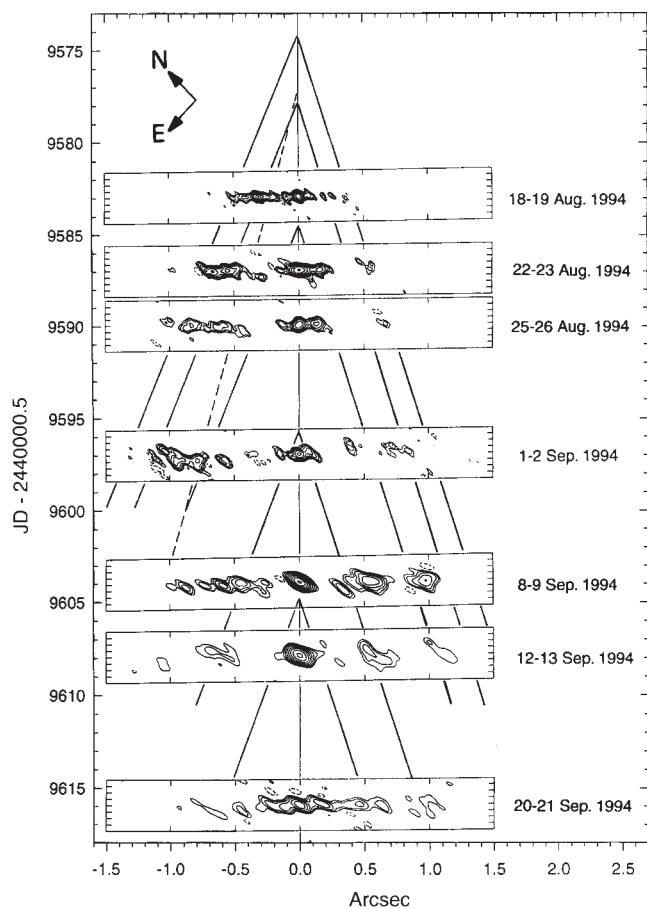


FIG. 4 A sequence of seven VLBA images of GRO J1655-40 at 1.6 GHz, each rotated anticlockwise by 43° , and each having an angular size of 3.0×0.4 arcsec. The vertical separation corresponds to the elapsed time between images, and each image is labelled with the date of observation. All images were deconvolved using the CLEAN algorithm; the resulting model was convolved with a gaussian fit to the beam, and the residuals restored, to produce these images. The data have been tapered to give east-west half-power beam-widths of 16, 21, 20, 25, 25, 39 and 43 mas respectively; the north-south beam-widths are a factor of ~ 2.5 larger, due to the southern declination of the source. Contouring is $S_0 \times 1.732^n$, with S_0 chosen to represent the peak-to-peak noise levels of 6, 4, 4, 1, 0.8, 3 and 1.5 mJy per beam. Peak flux densities are 429, 194, 104, 30, 69 and 16 mJy per beam, and total fluxes in the images are 3.4, 1.79, 1.23, 0.28, 0.20, 0.43 and 0.15 Jy, respectively. Solid lines (time lines) between the images identify motions of 54 mas d^{-1} on the left (NE) side and 45.5 mas d^{-1} on the right (SW) side. The dashed line indicates the 40 mas d^{-1} fit to the VLA data (see Fig. 3). The vertical line marks the central source, assumed to be the brightest point on each image, and the horizontal axis shows the spatial offset from that central source.

asymmetry must change from event to event. The changes in sidedness must be due to the physics of jet formation closer to the accretion-disk environment.

Because the jets lie nearly in the plane of the sky, the line-of-sight Doppler shifts (z) predicted by the kinematic model are dominated by the high value of the transverse Doppler shift, with the approaching jet having $z \approx 1.3$ and the receding jet $z \approx 1.8$. Thus lines from either jet, which are intrinsically in the visual spectrum, will be redshifted to the infrared, while ultraviolet lines will be redshifted into the visual.

As was first shown for SS443 (refs 11, 12), relativistic time-delay effects (which in this case shorten the period of the modulation as the jets move out) allow simultaneous determination of the jet velocity and the distance. Our kinematic model for GRO J1655-40 gives a distance of $3.2 \pm 0.2 \text{ kpc}$, in excellent agree-

ment with the 3.5 kpc reported by McKay and Kesteven¹⁷ and the 3.0-5.0 kpc distance reported by Tingay *et al.*⁵ on the basis of H I absorption spectra. For a distance of 3.2 kpc, the NE and SW proper motions of respectively 54 and 45 mas d^{-1} correspond to apparent superluminal and subluminal velocities of $1.05c$ and $0.88c$.

The VLBA image sequence, and the VLA images used in Fig. 3, cover two major radio flares, one peaking around day 9580, the other about day 9603. Both are associated with extensive periods of continuous jet ejection. As discussed above, the VLA data show three major ejection events associated with the first radio flare, on days 9578, 9584 and 9597 (Fig. 3). The VLBA images are consistent with these events, but show additional detail. In Fig. 4 one can see that the strong NE ejecta seen in the first four VLBA images correspond to the first major VLA event, here revealed to be a phase of nine days of nearly continuous ejections, beginning on day 9574 and reaching peak flux levels around day 9578. As mentioned before, this phase blends into the next VLA event; the corresponding ejecta are seen most clearly in the day 9597 VLBA image. But some of this emission, particularly to the SW, is not seen where expected from the earlier VLBA images. This is due in part to resolution effects, but some intrinsic change must also have taken place. The third and most long-lived VLA ejection event was traced back, using the VLBA images, to its origin on about day 9596, just before the fourth VLBA image. In this case the corresponding VLBA structures may be followed through all subsequent images, with only the expected weakening. There does appear to have been a significant gap between this and the preceding ejection events, although we cannot rule out ejecta that are relatively short-lived. We also note that the excellent agreement between the proper-motion path fitted to the data in Fig. 3, and the general proper motions of the structures seen in the VLBA images, implies that the NE ejecta on day 9597 were relatively compact.

The second major radio flare began at some time between days 9600 and 9608. The VLBA image of day 9604 is unique in showing the core as unresolved, the best direct evidence for

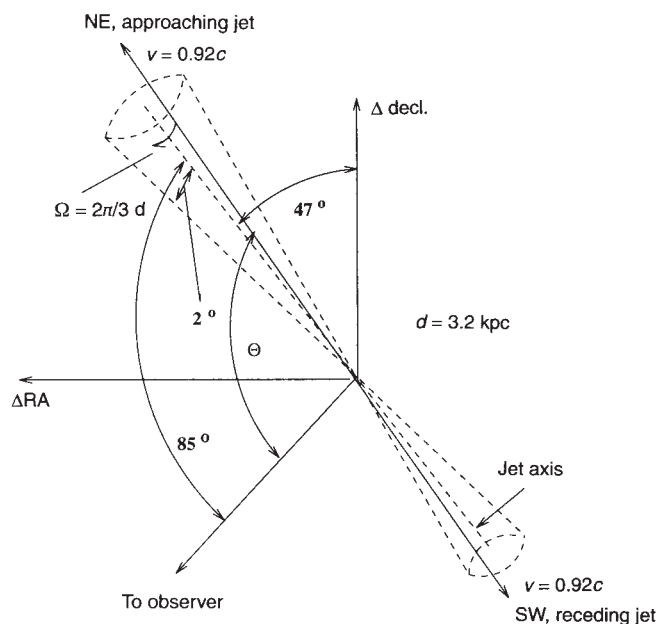


FIG. 5 The geometry of the kinematic model that fits the structure in the VLBA image sequence, showing two jets rotating clockwise about the jet axis, with a period of three days, with Θ being the angle of the jet axis with respect to the observer and $\Omega = 2\pi/3 d$ the angular rotation rate. The jets maintain an angle of 2° to the jet axis, which is inclined 85° from the line of sight with a position angle of 47° . The jet velocity is $0.92c$ and the distance is 3.2 kpc. RA, right ascension; decl., declination.

several days with no jet ejection preceding the flare. The ejecta corresponding to the second radio flare are first seen (see also the timelines in Fig. 4) as a resolved core on day 9608, and by day 9616 both these and subsequent ejecta are obviously extended. Again the indications are that the radio flare is associated with continuous ejection over at least 10 days. The ejecta nearest the core in the images for days 9608 and 9616 also represent the only case where the jets appear to be nearly symmetric.

Discussion

Relation of radio to X-ray emission. The relationship between the radio and X-ray lightcurves of GRO J1655–40 is discussed in detail by Harmon *et al.*³; here we merely comment on the timing of ejection of major radio-emitting structures. The sequence of VLBA images shows that the first two radio flares in Fig. 1 are characterized by extended periods of continuous ejection of synchrotron-emitting plasma, with that ejection beginning on days 9574 ± 1 and 9605 ± 2 , respectively. The corresponding beginning, peak and end (B, P and E in Fig. 1) of the first and second X-ray events occurred roughly at days (9561, 9570, 9581) and (9600, 9606, 9612). Therefore the first ejection event started a few days after the X-ray flux began to decay, with the radio peak (day 9580) roughly matching the end of the first X-ray flare. The ejection leading up to the second radio peak took place just before the maximum of the X-ray emission, and again the radio peaked a few days before the end of the X-ray event.

The third radio flare in Fig. 1 coincides roughly with a third X-ray flare, which was immediately followed by a fourth X-ray flare. Weeks later the radio emission could be resolved by the VLA, and extrapolation of VLA images on day 9704, showing a 1.8-arcsec double radio source, gives an ejection date for the third radio flare of day 9668 ± 5 .

The radio jets in the accretion disk environment. The “wiggles” in the otherwise highly collimated radio jets seen in the VLBA images in Fig. 4 may be due either to internal structure, or to rotation of the jets about a jet axis at an angle of 2° with a period of three days. The latter case, which is consistent with the data in the VLBA images, means a very short period compared to the 162.5-d period for the SS433 jets^{10–15} which has been attributed to precession of the accretion disk. If the three-day period reflects precession, the binary may have an extremely short period, of the order of several hours. The three-day period may instead be associated with the orbital period of the binary. Then, depending on whether one adopts models wherein the jet axis is the axis of the accretion, or models where it is defined by the spin axis of the compact object, the 2° is a measure either of the warping of the accretion disk by the companion star, or of the inclination of the compact object's spin axis with respect to the accretion disk and the orbital plane. If the radio jets leaving the stellar system are produced by magnetohydrodynamical or gas-dynamical jets on the axis of the accretion disk, a warp in that disk caused by the companion star might explain the basic kinematic behaviour. Should the jets instead originate from the immediate environment of the compact

object, one would need to invoke a mechanism for a 2° misalignment between the axis of the compact object and that of the accretion disk/orbital plane. Independent of whether the wiggles are due to kinematics or not, the jet axis is roughly perpendicular (85°) to the line of sight; and as the axis of the accretion disk would then be $\leq 2^\circ$ from the axis of the orbital plane, the system may be an eclipsing binary, as suggested by the possible eclipse event reported by Bailyn *et al.*⁷.

Implications for other sources. GRO J1655–40 is a important object in at least five respects: the timing of its X-ray and radio flares indicate a close connection between changes in the accretion disk and radio jet production, it has highly collimated relativistic jets, it is nearby, it is bright, and it evolves quickly. This combination makes it the best known system for studying relativistic jets from accretion disks. With $\gamma \approx 2.5$, the jets are relativistic enough for their physics to be directly analogous to that of extragalactic jets. GRO J1655–40 has already demonstrated several important points. First, relativistic jets can be intrinsically asymmetric, and the sense of that asymmetry may vary with time. Second, individual ‘blobs’ of emission, in this source at least, appear to be associated with well defined episodes of continuous jet ejection, which generally produce emission on both sides of the central engine. Third, the proper motions derived from observations which do not resolve those ‘blobs’ may be systematically in error, because of the combination of flux densities which fade with time and beam smearing. Fourth, the ejecta have lifetimes ranging from a few days to several months, and jet ejection occasionally seems to halt completely, even as the central source remains bright. Whether the latter is due to extremely short-lived ejecta or reflects some genuine, permanent core of emission is still unclear.

GRO J16755–40 is the third Galactic star system shown to have relativistic jets, the others being SS433 (refs 10–15) and GRS1915+105 (ref. 16) with jet velocities of $0.26c$ and $0.92c$, respectively. One indication that these three may not be the only star systems with optically thin jet ejection is the similarity of the first two radio flares in GRO J1655–40 (Fig. 1) to initial flare events of other Galactic X-ray transients (A0620–00 (refs 18, 19), GS2000+35 (refs 19, 20), GS2023+338 (refs 8, 19), GS1124–683 (refs 19, 21), Centaurus X-4 (refs 19, 22) and Aquila X-1 (ref. 19). These flares were fitted¹⁹ by models which involved the spherical expansion of relativistic plasma; they may instead have been highly collimated, jet-ejection events. Despite some similarities to these eight objects, GRO J1655–40 is unique, both in its X-ray properties and in the character of its radio emission. At high energies, its X-ray and γ -ray fluxes are unusually intense and unusually variable³; at radio wavelengths, the flux density is again very high, and both the observed proper motion and the inferred intrinsic velocity ($0.92c$) are extraordinary.

Note added in proof: Bailyn *et al.*²³ recently reported that GRO J1655–40 is an eclipsing and spectroscopic binary system with a period of 2.62 days, consistent with the uncertainty in our three-day period, and that the invisible primary star is a black hole with a mass between 4 and 5.2 solar masses. □

Received 17 February; accepted 2 May 1995.

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ACKNOWLEDGEMENTS. We thank the large number of VLA observers who generously gave us some of their observing time to observe GRO J1655–40. Special thanks are due to the VLBA operations staff for their timely execution of the VLBA observations and correlations, allowing excellent coverage of the radio flares in GRO J1655–40, and the rapid calibration and imaging (within 10 days of the first VLBA observation) which indicated the importance of subsequent observations. The National Radio Astronomy Observatory is operated by Associated Universities, Inc., under a cooperative agreement with the US NSF.

Evidence for a large undetected population of dust-reddened quasars

Rachel L. Webster*, Paul J. Francis*,
Bruce A. Peterson†, Michael J. Drinkwater‡
& Frank J. Masci*

* School of Physics, University of Melbourne, Parkville, Victoria 3052, Australia

† Mount Stromlo and Siding Springs Observatory,
The Australian National University, Private Bag,

Weston Creek Post Office, ACT 2611, Australia

‡ Anglo-Australian Observatory, Coonabarabran, NSW 2357, Australia

QUASARS have been detected at many wavelengths, but often ones that are bright at one wavelength are very faint or undetectable at other wavelengths. It has therefore been impossible to design a single search technique that would identify all quasars, raising the question of how many may have gone unidentified. Here we show that quasars selected from a radio catalogue have a wide range of optical colours, which we interpret as arising from varying amounts of dust along the line of sight. Most of this dust probably lies within the quasar host galaxy. If the radio-quiet quasars that would normally be detected optically contain as much dust as the radio-loud ones (and have gone undetected at other wavelengths), then 80% of them have been missed by optical surveys. These missing quasars could adequately account for the observed X-ray background.

Possible obscuration and absorption processes in quasars mean that the ideal search wavelength is in the radio band. Previous radio surveys, however, have either been incomplete or contained only a small fraction of quasars. We are studying a subset of the Parkes catalogue¹, chosen to have flat radio spectra ($\alpha > -0.5$ where flux $S_\nu \propto \nu^\alpha$), and 2.7-GHz fluxes $S_{2.7} > 0.5$ Jy. Regions with 20° of the Galactic plane are excluded, and the survey region covers declinations from $+3^\circ > \text{dec.} > -45^\circ$. A total of 323 sources satisfy these selection criteria and a full description of the sample will be published elsewhere. This sample contains a higher fraction of quasars than any existing flux-limited radio-selected sample, presumably due to its low flux cut-off and flat radio spectra.

Of the 323 sources, 218 have been spectroscopically identified as quasars, 37 as other objects such as galaxies and planetary nebulae, 18 have been called BL Lacs and 50 have no spectral identification at present. Accurate radio positions were obtained with the Australia Telescope Compact Array to enable optical and near-infrared identifications. Imaging in the K band to $K \approx 19.5$ mag was obtained with the IRIS camera on the Anglo-Australian Telescope, and the CASPIR and PICNIC cameras on the Mount Stromlo and Siding Springs Observatory 2.3-m telescope, both for a sample of 26 sources which had no spectral identification and $B_J \geq 22.5$, and for a random subset of 59 other Parkes quasars. An additional 52 K-band magnitudes (K -magnitudes) were obtained from the literature²⁻⁵, including 11 for BL Lacs. A comparison sample of 26 optically selected quasars drawn from the Large Bright Quasar Survey (LBQS)⁶ was also observed in the K band with the NICMOS camera on the Steward Observatory 1.5-m telescope. Photographic B_J magnitudes were obtained from COSMOS and APM scans of UK Schmidt survey plates.

In Fig. 1a we plot $B_J - K$ colours for the quasar in the Parkes sample as function of K magnitude. A striking feature is the enormous scatter in $B_J - K$ colours, with the range $1 < B_J - K < 8$. The optically selected quasars show a much smaller colour range.

Is the enormous spread in the $B_J - K$ colours of the Parkes quasars intrinsic, or due to dust somewhere along the line of

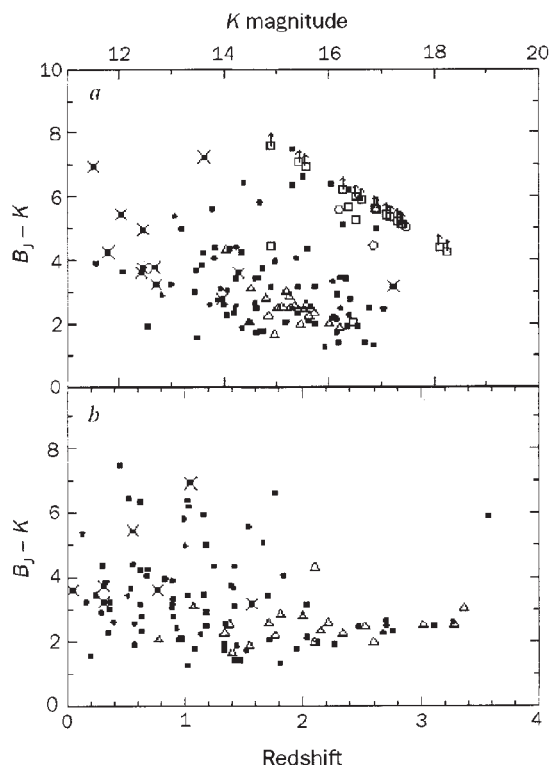


FIG. 1 a, Plot of $B_J - K$ colours as a function of K magnitude for 97 Parkes sources (squares: filled, with redshift; open, without redshift; 48 quasars from Wright *et al.*¹ (circles: filled, with redshift; open, without redshift; crossed, BL Lacs) and 26 quasars from the LBQS sample (open triangles). b, Plot of the distribution of redshift with $B_J - K$ for those sources of known redshift.

sight? If we extrapolate the continuum shapes of the reddest quasars out to the ultraviolet, we find that they have ratios of ionizing photon flux to observed B-band continuum flux smaller than those of the bluest quasars by factors of >100 . As Fig. 2 shows, however, the equivalent widths of the photoionized emission lines do not anticorrelate with the redness of the continuum. This implies that the emission-line region is exposed to a much

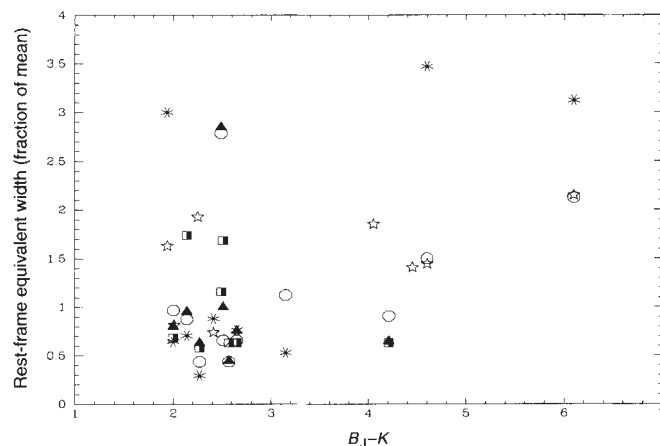


FIG. 2 Plot of equivalent widths of five broad lines as a function of K magnitudes. Solid triangles are $\text{Ly}\alpha$, half-filled squares for N v, circles for C iv, asterisks for C iii and hollow stars for Mg ii. Data are taken from Wilkes²¹; all equivalent widths are normalized by the mean value for that line.

harder continuum than that detected on Earth, strong evidence for line-of-sight dust.

Further evidence for dust comes from the spectral energy distributions of two typical red quasars for which we have obtained optical spectra. These spectral energy distributions are shown in Fig. 3, compared with a composite optically selected quasar spectrum⁷. Reddening the optically selected spectrum using an extinction law⁸ with dust located at the quasar redshift, we can closely match the spectra of the two red quasars. In another case, a low-redshift quasar Q1706+006, where we observe both the H α and H β lines, the Balmer decrement is equivalent to four magnitudes of extinction, consistent with the observed $B_J - K \approx 7.5$. We therefore conclude that the scatter in $B_J - K$ colours is predominantly caused by different dust extinction along the lines of sight to quasars. The lower envelope of the colour distribution, $B_J - K \approx 2.5$, would thus represent the intrinsic colour of most quasar continua. This colour corresponds to an optical continuum slope of $f_\nu \propto \nu^\alpha$, where $\alpha \approx -0.3$, which is the slope predicted by the free-free continuum radiation model⁹.

Why do the optically selected quasars show such a narrow range of $B_J - K$ colours, lying at the lower envelope of the $B_J - K$ distribution of the Parkes quasars? One possibility is that radio-quiet quasars lack line-of-sight dust. But even if optically selected quasars have as much line-of-sight dust as the Parkes quasars, we would not expect to detect many red quasars optically. The reason is as follows: consider an optical quasar survey with a limiting magnitude m in the B band. For a quasar reddened by 5 magnitudes in B to be included in the sample, its intrinsic B band magnitude would be $m - 5$. The bright end of the quasar luminosity function is steep, so quasars with intrinsic B magnitudes of $m - 5$ have surface densities $\sim 10^7$ smaller than quasars with intrinsic B magnitudes m (ref. 10), making their detection highly improbable.

We use a simple model to predict the colour distribution of LBQS quasars. We assume that the real distribution of optically selected quasar colours is the same as a subsample of 42 Parkes quasars which is complete in a limited range of right ascension, (included in Fig. 1). We then determine the numbers of quasars which would satisfy the LBQS selection limit in the B band. We have modelled this selection in Fig. 4. The model correctly predicts that the colours of optically selected quasars should show a small dispersion, and be clustered strongly around the lower envelope of the distribution of colours of the radio-selected sample. Furthermore, it predicts that for every quasar seen with a given B-band magnitude, there are about five more with the same intrinsic B magnitude but reddened below the LBQS survey magnitude limit.

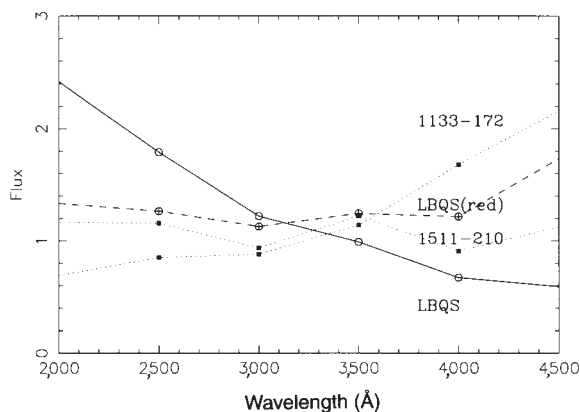


FIG. 3 The relative flux distribution as a function of wavelength for the mean LBQS spectrum⁶ (solid line), a reddened LBQS spectrum (dashed line) and two 'typical' reddened Parkes quasars (dotted lines). Details are given in the text.

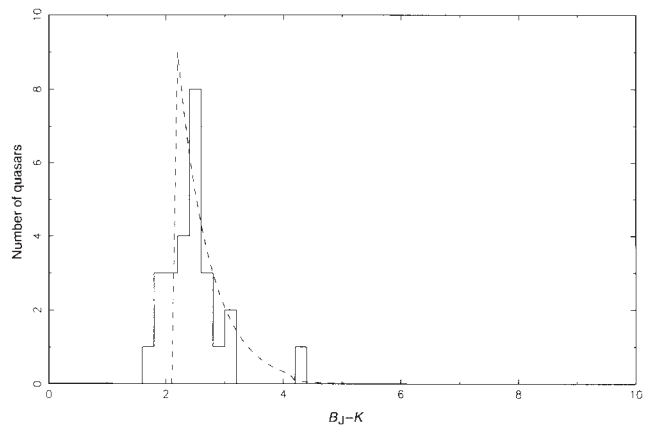


FIG. 4 The colour distribution of LBQS quasars predicted by our simple model (dashed line), compared to the histogram of the observed values.

We note that the colours of the unidentified sources fall at the red end of the distribution of colours of Parkes quasars. Their radio spectral slope distribution is indistinguishable from that of the identified quasars. Because in all cases both the K band and radio images are point sources, we suggest that the previously unidentified sources are predominantly very red quasars, which would further strengthen our conclusions.

Other evidence for dust can be found in X-ray selected samples. Stocke *et al.*¹¹ find a factor of 100 dispersion in the X-ray to optical flux ratios of their sample of X-ray selected quasars, whereas McDowell *et al.*¹² find that X-ray to optical ratios correlate with $B - K$ colours, both of which might be also due to variable dust extinction. Most importantly, this large scatter in X-ray to optical ratios is seen in both radio-loud and radio-quiet quasars. Other studies at optical and infrared wavelengths also find evidence for dust¹³⁻¹⁵.

The dust might be located in intervening galaxies or protogalaxies^{16,17}, distributed throughout the quasar host galaxy, or in a dusty torus close to the quasar nucleus. Models of dusty intervening galaxies predict the fraction of reddened quasars to increase with redshift. In Fig. 1b we plot $B_J - K$ colours against known redshifts. The $B_J - K$ colours for both the Parkes and the LBQS sources do not correlate with redshift, indicating that the scatter cannot be due to redshift-dependent evolution. However the redshift distribution shown does not include most of the reddest, faintest sources, which will probably contain the highest redshift objects. An example of a dusty host galaxy is given in Hubble Space Telescope images of the BL Lac object 1413+135 (ref. 18), which shows strong evidence that the host galaxy is an edge-on spiral. Wills *et al.*¹⁹ have presented an example of the third possibility, where the quasar would be viewed through the edge of the dusty torus, causing the optical image to be reddened.

Recent theoretical models of the contribution of active galactic nuclei to the X-ray background²⁰ have included both an absorbed and an unabsorbed population of active galactic nuclei, where the absorption is due to gas in a torus near the nucleus. A population with a ratio of 2-3 of absorbed to unabsorbed active galactic nuclei provides a good fit, not only to the observed spectrum of the X-ray background, but also to the source counts in both soft and hard X-ray bands. These calculations are consistent with our results.

We conclude that many flat-spectrum radio-loud quasars are strongly obscured by dust at optical wavelengths. If radio-quiet quasars have similar amounts of dust, as X-ray observations suggest, existing surveys of bright optical quasars may be missing $\sim 80\%$ of quasars with a given intrinsic optical magnitude. The intrinsic quasar spectrum is therefore given by the blue envelope of the observations, which has a slope of $f_\nu \propto \nu^{-0.3}$ consistent with the predictions of the free-free model. It is likely that most

of the dust lies within the quasar host galaxies, but our data are consistent with some reddening due to dust located in intervening systems. $\square$

Received 16 November 1994; accepted 4 May 1995.

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Identification of faint radio sources with optically luminous interacting disk galaxies

R. A. Windhorst*, E. B. Fomalont†, K. I. Kellermann†, R. B. Partridge‡, E. Richards‡, B. E. Franklin*, S. M. Pascarelle* & R. E. Griffiths§

* Department of Physics and Astronomy, Arizona State University, Tempe, Arizona 85287-1504, USA

† National Radio Astronomy Observatory, Edgemont Road, Charlottesville, Virginia 22903-2475, USA

‡ Haverford College, Department of Astronomy, Haverford, Pennsylvania 19041, USA

§ Bloomberg Center for Physics and Astronomy, Johns Hopkins University, Baltimore, Maryland 21218-2695, USA

THE question of when galaxies and stars started to form in the early Universe is one of the most fundamental in cosmology. Faint radio sources (with fluxes of a few microjanskys or less) may be these early galaxies; radio surveys have shown them to be increasingly common as fainter levels are reached^{1–7}, suggesting that they are an important constituent of the early Universe. Many of these sources were identified with faint blue galaxies—some of which have peculiar morphology (perhaps arising from interactions)^{1,8–10} or with galaxies hosting enhanced star formation activity^{10–12}, but lack of optical resolution has made the nature of these identifications rather uncertain. Here we present the results of a very deep radio survey, obtained with the Very Large Array, of a field imaged with the Wide Field Camera on the Hubble Space Telescope. We find that most of the microjansky sources are indeed faint blue galaxies, with light profiles resembling disk galaxies. More than half of these galaxies occur in pairs or small groups, compared to fewer than ten per cent of nearby galaxies. We conclude that the microjansky sources are luminous disk galaxies, in a starburst (or post-starburst) phase that was triggered by interactions or mergers. Only a few show evidence for an active nucleus^{2,13,14}, which is the other possible power source for the emission.

To be suitable for deep radio imaging, a field imaged by the Hubble Space Telescope (HST) should contain no “strong” sources with gigahertz radio flux $S \geq 1$ mJy. One such deep HST

field is available at (J2000) right ascension 13 h 12 min 17.4 s, dec. $+42^\circ 38' 06''$, and was imaged in May 1992 as part of the HST Medium Deep Survey key project^{15,16}. Even before its refurbishment, the HST could provide images with a resolution better than 0.2 arcsec full-width at half-maximum (FWHM) after careful deconvolution, but only with limited dynamic range ($\lesssim 4$ –5 mag)^{17–19}. As the median scale-length of faint field galaxies ($V \lesssim 25$ mag) is ~ 0.4 arcsec (refs 16, 20), and the HST sky-brightness is 2–3 mag darker than from the ground¹⁷, deep HST images allow detection of faint galaxies down to $V \lesssim 26$ and $I \lesssim 25$ mag, and at the same time allow the study of the kiloparsec-scale structure of galaxies at redshift $z \geq 0.2$ at $\lesssim 0.2$ -arcsec resolution (see Fig. 1 of ref. 15), which cannot be done over wide fields even from the best ground-based sites.

The HST images were obtained with the Wide Field Camera (WFC) in parallel observing mode. A total of six V-band exposures were made in 3.5 h, and 14 I-band exposures in 8.1 h. We processed the WFC images as described previously^{15,18,21}. The 2 σ surface brightness sensitivity of the WFC mosaics is ~ 27.2 mag arcsec⁻² in V and ~ 25.9 mag arcsec⁻² in I (ref. 18). The 3 σ point-source sensitivity (including the effects of spherical aberration) is¹⁸ ~ 26.4 mag in V and 24.8 mag in I . We deconvolved the WFC images with the modified Lucy algorithm, using several spectroscopically confirmed stars in the WFC field as point-spread functions¹⁵. The resulting WFC dynamic range is limited to $\sim 100:1$, which, however, is adequate for our faint galaxies. The deconvolved V-plus I-band mosaics are shown in Fig. 1.

The 8.44-GHz Very Large Array (VLA) observations have a field-of-view of 5.25 arcmin (half power beam width, HPBW), and were centred at the HST field¹⁵. From October 1993 to January 1994, we collected 84 h of good quality data in the VLA D-configuration. The data were edited, imaged, and deconvolved using the CLEAN algorithm (refs 2, 5, 6). The observed image noise is 2.0 μ Jy r.m.s., in good agreement with the expected value of 1.9 μ Jy. From November 1994 to January 1995, we observed the same field for an additional 75 h in the C-configuration with a noise of 2.0 μ Jy. The combined C + D-configuration image has a noise of 1.5 μ Jy and is the deepest radio survey made to date. The negative side of the noise histogram in the images showed no deflections below -7.6 μ Jy (5.1σ), suggesting that all sources with 8.44-GHz flux density $S_{8.44} \geq 8.8$ μ Jy (5.9σ) in the combined image each have a $\geq 99\%$ probability of being real. The dynamic range of the observations—limited by residual instrumental and atmospheric phase errors—is at least 140:1, so that the image is not dynamic-range limited. Because $\sim 40\%$ of previously observed microjansky sources have² angular sizes > 5 arcsec, we derive flux densities from the D-configuration image (10 arcsec FWHM), de-blending a few confused sources using the higher-resolution combined C + D-configuration image. The D-configuration image is shown as contours in Fig. 1. All positions come from the C + D-configuration image (3 arcsec FWHM) to allow unambiguous identifications.

There are 24 radio sources in the VLA field located inside the HST mosaic (Fig. 1 and Table 1). Of these, 19 have $S_{8.44} \geq 8.8$ μ Jy ($\geq 4.5\sigma$ in the D- and $\geq 5.9\sigma$ in the combined C + D-configuration image). Of the remaining five objects, sources 20 and 21 were detected below 8.8 μ Jy, source 23 was discarded as part of the radio-optical sample because it is at the poorly exposed WFC mosaic edge, and sources 14 and 22 are upper limits at the position of known bright HST galaxies. Including these latter two, almost all galaxies brighter than $V \approx 23$ mag have radio counterparts or meaningful upper limits (two cases) at the microjansky level, as expected from their radio-optical spectral-index distribution that peaks^{1,8,22} around $\alpha_{ro} \approx 0.14 \pm 0.23$.

We measured coordinates for WFC objects and registered the VLA contours on top of the WFC mosaic to within ~ 0.2 arcsec. The 3-arcsec (FWHM) VLA C-configuration beam yields positions of 0.1–0.5 arcsec accuracy. We analysed the optical identi-

fication statistics with the likelihood ratio method, which is based on the normalized radio-optical position differences or m -statistic²³, and produces a list of optical identifications similar to that produced by the m -statistic²⁴. The confidence level for individual identifications is indicated by $P(\text{id.})$, the probability that they are the correct identification given the combined radio-optical error circles and the surface density of contaminating objects. $P(\text{id.})$ ranges from 86 to 99%. All objects with $S_{8.44} \geq 8.8 \mu\text{Jy}$ and $P(\text{id.}) > 86\%$ in Table 1 form the radio-optical subsample used for our study. The optical identification statistics are as follows. Of the 19 sources in the radio-optical sample, four (sources 5, 7, 11 and 12) have no optical candidate with $P(\text{id.}) > 86\%$ down to $I \approx 25.3$ mag, and 15 have a likely candidate, including ~ 1.1 expected contaminating objects. So

the identification sample has $\sim 7\%$ contamination, the average sample reliability is 93%, and the sample completeness is nearly 100%. The identification fraction corrected for contamination is $73 \pm 10\%$. Deep surveys (using charge-coupled device (CCD) detectors) down to $V \approx 26.5$ mag have identified nearly 100% of the millijansky radio source population¹⁰ with a peak in the magnitude distribution around $V \approx 24$ mag. The somewhat lower identification fraction of the microjansky population down to an HST image of similar depth is interesting. The unidentified objects, although only few in number, may be obscured internally, and/or by dust at very large redshifts^{10,25}.

Of the 15 objects with a likelihood ratio ≥ 2 , objects 4 and 17 are quasars, four are isolated galaxies (sources 8, 13, 15 and

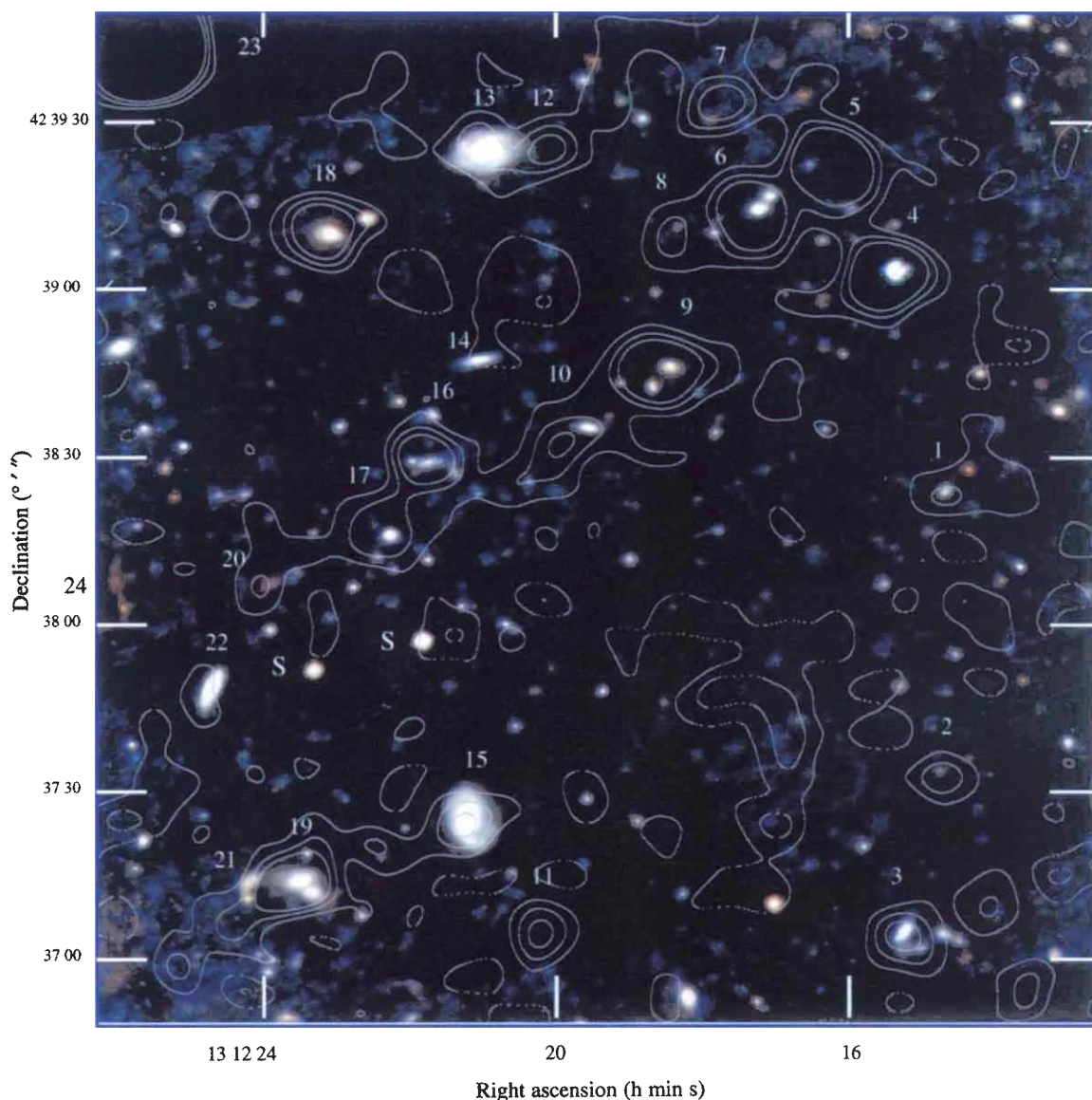


FIG. 1 Colour image of the 8.1-h cycle 2 mosaic with the Hubble Space Telescope Wide Field Camera in the I band (F785LP filter with $\lambda_{\text{eff}} = 8,920 \text{ \AA}$, red gun) and 3.5 h in the V band (F555W with $\lambda_{\text{eff}} = 5,420 \text{ \AA}$, blue gun; $(V+I)/2$ is in the green gun). The mosaic shown is 160×160 arcsec, but individual WFC orbits were dithered by ± 30 arcsec, so that only the central 100×100 arcsec has the full exposure time, while the noise increases towards the edges. The HST images were deconvolved in subsections with the Lucy method, using various stars in the field. The WFC stack has $\lesssim 0.2$ arcsec resolution (FWHM),

a 3σ point source sensitivity of $R \approx 25.6$ mag (with spherical aberration), and a 2σ surface brightness sensitivity of $R \approx 26.5$ mag arcsec⁻². The VLA image is indicated by contours at $-6.0, -3.0$ (dashed lines), $+3.0, +6.0, +9.0$ (full lines) μJy . The radio image is attenuated by the VLA primary beam, which is approximately gaussian with 5.25 arcmin HPBW. Spectroscopically confirmed stars are labelled 'S', the two microjansky quasars at $z = 2.561$ are objects 4 and 17. Galaxy 24 is just off the edge of Fig. 1. Note the fraction of double, interacting, and/or galaxies in small groups among the microjansky identifications.

TABLE 1 HST surface photometry of microjansky radio sources in the 13 h +43° VLA field

Source	h	min	s	RA* °	Decl.* °	P(id.)†	S _{8.4} ‡ (μJy)	V _{int} (mag)	I _{int} (mag)	z	r _e /r _s ¶ (")	PA# (°)	Ell.☆ (1 - (b/a))	Comments
1	13	12	14.58	+42 38	22.0	0.91	09.8	23.44	21.81		—/0.43	146	0.30	Galaxy in group
			14.45		21.6									
2	13	12	14.68	+42 37	31.5	0.89	08.8	26.3	25.2		1.9/1.01	161	0.35	Galaxy in pair
			14.51		31.1									
3	13	12	15.20	+42 37	02.7	0.95	12.1	20.74	19.71	0.322	2.6/0.62	151	0.55	Galaxy in compact group
			15.08		02.6									
4	13	12	15.30	+42 39	00.7	0.99	27.3	18.42	17.79	2.561	<0.2	—	—	Quasar with fuzz?
			15.29		00.9									
5	13	12	16.50	+42 39	21.5	0.24	31.8	25.1	24.4		—/0.37	161	0.15	Galaxy in group, but 5" E
			16.07		21.3									
6	13	12	17.26	+42 39	12.3	0.97	23.2	22.22	20.05		—/1.10	081	0.8	Galaxy in pair
			17.17		12.5									
7	13	12	17.59	+42 39	27.9	0.04	15.2	26.1	24.8		0.2/0.79	001	0.2	Faint galaxy in group, but 3" S
			17.59		30.6									
8	13	12	17.91	+42 39	09.5	0.86	15.3	>26.5	25.2		2.1/0.76	071	0.3	Faint galaxy
			18.30		08.1									
9	13	12	18.43	+42 38	44.0	0.98	21.8	23.17	20.28		—/0.94	169	0.65	Galaxy in pair or group
			18.46		43.9									
10	13	12	19.62	+42 38	31.7	0.87	17.8	22.25	20.45		—/0.45	081	0.5	Galaxy in pair
			19.57		33.0									
11	13	12	20.16	+42 37	03.4	—	09.6	>26.5	>25.3		—/—	—	—	No identification
			20.16		03.4									
12	13	12	20.51	+42 39	23.8	0.07	11.8	>26.5	25.3		—/—	—	—	Very faint object in spiral arm of 13?
			20.02		23.4									
13	13	12	21.06	+42 39	22.5	0.92	10.1	19.18	17.98	0.302	—/1.41	101	0.30	Barred spiral galaxy
			21.04		23.3									
14	13	12	21.24	+42 38	45.1	0.82	≤4§	21.25	20.26	0.126	—/1.03	101	0.8	Edge-on disk galaxy
			21.32		45.0									
15	13	12	21.31	+42 37	23.1	0.95	10.1	19.05	17.80	0.180	—/2.70	011	0.45	Barred spiral galaxy
			21.33		22.9									
16	13	12	22.00	+42 38	27.1	0.93	16.6	24.33	22.72		—/1.22	101	0.6	Galaxy in compact group
			21.83		27.3									
17	13	12	22.39	+42 38	13.9	0.91	11.5	21.03	19.57	2.561	<0.2	—	—	Quasar in group
			22.49		14.1									
18	13	12	23.25	+42 39	07.5	0.94	22.6	21.60	19.29		—/0.78	086	0.45	Galaxy in pair
			23.24		08.6									
19	13	12	23.62	+42 37	11.5	0.98	24.4	20.58	18.76	0.401	—/0.52	096	0.25	Double galaxy in group
			23.67		11.9									
20	13	12	24.26	+42 38	03.5	0.87	§8.3	25.5	24.4		—/0.94	121	0.6	Faint galaxy in group
			24.11		05.0									
21	13	12	24.42	+42 37	11.8	0.05	§≤7	22.50	20.60		—/0.79	010	0.45	Galaxy in group at edge, but 7" N
			24.42		05.2									
22	13	12	24.89	+42 37	46.7	0.71	§≤4	19.07	17.55	0.179	1.5/0.93	156	0.6	Edge-on disk galaxy
			24.99		46.5									
23	13	12	25.74	+42 39	37.3	—	380.0	—	>21.3		—/—	—	—	Object at poorly exposed edge
			25.74		41.5									
24	13	12	27.36	+42 38	01.2	0.90	22.4	22.00	19.85		—/0.60	171	0.55	Edge-on disk galaxy
			27.50		00.3									

* First line for each source indicates the right ascension and declination (Equinox J2000.0) of the HST galaxy core. The second line gives the radio position from the combined C + D-array image (3" HPBW). The radio reference frame is within 0.1" of the calibrator 12244 + 408³⁰. Likely id.s can have offsets up to 1–2", as the HST galaxy scale-lengths can be that large^{15,16,18,20}, and the disk radio emission does not have to coincide with the optical galaxy core².

† Probability that the object is the correct identification, P(id.), from the radio–optical position difference distribution and the Poisson distribution of contaminating objects, using the observed galaxy density and the likelihood ratio method²³.

‡ Total 8.44 GHz sky-flux corrected for VLA primary beam from the D-array image, but in a few cases de-blended from the combined C + D-array image. An § indicates objects not in the radio sample with S_{8.44} ≥ 8.8 μJy (5.9σ).

|| Integrated total V- and I-magnitudes from the undeconvolved images, using the WF/PC zero points¹⁵.

¶ deVaucouleurs half-light radius r_e and exponential disk scale-length r_s, respectively, of the best fit to the bulge or disk part of the deconvolved WFC image. If no value for r_e is quoted, a meaningful bulge could not be fitted.

Sky position angle (from north to east) of the outer semimajor axis, corrected for the HST spacecraft orientation.

☆ Average best-fit ellipticity of the outer disk where a is the major, and b the minor, axis.

24), and nine (sources 1, 2, 3, 6, 9, 10, 16, 18 and 19)—or 60 ± 13% of the identification sample—coincide with double galaxies, compact groups, or interacting/merging galaxies (see Fig. 1). This is to be compared to 7 ± 2% of the nearby galaxies seen in pairs or groups²⁶, and 15–34% of field galaxies seen^{26,27} in pairs or groups in several HST fields at z ≈ 0.5. Hence, the microjansky population at z ≈ 0.5 sees more pairs than present in nearby field galaxy samples, and probably at least as many, if not more than

HST sees in the field at z ≈ 0.5. Galaxies in pairs or double/interacting galaxies occupy <1.5% of the total WFC area²⁶; ~0.3% of the WFC area is covered by the combined 2σ radio-optical error circles, so that the number of double galaxies identified by chance down to I ≤ 25 mag is ≤ 0.3 objects. We note that our sample and the area covered are still very small, so that statistical variations in the redshift distribution through which we are looking could be substantial².

We made elliptical isophote fits to the galaxy surface-brightness profiles, which allows us to determine unbiased scale-lengths at 0.2-arcsec resolution (FWHM)^{15,18,21}. Spectra obtained with the Multiple Mirror telescope (MMT) were used to measure redshifts out to $z \approx 0.4$ for nine of the brighter objects with $I \leq 20.3$ mag, details of which are given elsewhere¹⁵. Results on all objects are summarized in Table 1. In January 1995, Lowenthal and Koo (personal communication) obtained redshifts with a long integration on the Keck telescope for another 6–8 galaxies down to $I \leq 22.5$ mag with measured redshifts up to $z \approx 0.7$. For three radio sources in the sample (13, 15, 19), as well as galaxies 14 and 22, HST light-profiles have been published¹⁵. They are dominated by an exponential disk. Of the remaining objects, galaxies 1, 6, 9, 10, 16, 18 and 24 are also dominated by exponential profiles. Galaxy 8 is too faint to be classified. Only galaxies 2 and 3 show a significant $r^{1/4}$ contribution to their light-profiles, and contain a clear bulge embedded in a disk. Hence, at least 67% of the microjansky sample consists of disk-dominated galaxies, generally with little or no colour gradients^{15,19}. The observed range of exponential disk scale-lengths is $0.4'' \leq r_s \leq 2.7''$ with median $r_s \approx 0.8 \pm 0.2$ arcsec, somewhat larger than the median HST scale-length of 0.4 arcsec for faint field galaxies^{16,20}, indicating that our radio galaxies may be more luminous than ordinary field galaxies, as the absolute luminosity of galaxies is known to be proportional to their scale-length^{18,20}. This is substantiated by the Hubble diagram for the few microjansky identifications with available redshifts: they have absolute luminosities $M_V = -22.2$ mag, comparable to those of sub-millijansky and millijansky galaxies²⁸, but ≥ 0.7 mag brighter than typical field galaxies²⁰, so the microjansky population does not (yet) sample a large fraction of dwarf-like luminosities.

Two microjansky sources coincide with weak quasars, consistent with the fraction of active galactic nuclei (AGN) seen at brighter radio fluxes⁸. Both quasars are at the same redshift of $z = 2.561$ and are ~ 1.5 arcmin apart, which rules out a gravitational lens, but suggests that a group or cluster at $z \approx 2.56$ may be present in the field. The average ($V-I$) colour of the microjansky radio galaxies in Table 1 is 1.5 ± 0.7 mag, similar to that of a much larger sample of HST field galaxies (with $V \approx 20$ –24 mag)^{16,20}. Five radio galaxies (6, 9, 18, 19 and 24) have colours as red as the upper envelope in the ($V-I$) colour-magnitude diagram of HST field galaxies^{16,18,20}. Four of these are located in pairs or groups, and may be post-starburst galaxies as seen in distant clusters²⁹. The eight remaining galaxies in Table 1 have colours bluer than the red upper envelope of field galaxies^{16,20}. Five of these also occur in pairs or groups. These are possibly galaxies with enhanced star-formation, induced by companions or interactions. The MMT spectra of the blue galaxies 3, 13, 15 and 19 have weak narrow emission lines (O II 3,727 Å and H α of typical rest-frame equivalent width $W_\lambda \approx 20$ –30 Å), suggesting star-formation.

Are the microjansky galaxies dominated by starbursts and/or by weak AGN? The flat high-frequency radio spectra² suggest possibly free-free or thermal dust-emission in extended disks², and/or compact synchrotron cores. The 60% of double or interacting identifications from the HST images, and the 67% of exponential disks among the HST identifications, suggest that the microjansky sources occur in a disk-dominated galaxy population that is undergoing interactions, with optical luminosities that generally exceed L^* . Combined with the fact that only 20–30% of microjansky sources are variable on timescales of years^{2,13}, but that $>40\%$ are extended radio sources² (≥ 5 arcsec), our HST results suggest that the majority of the microjansky population is likely to be of a starburst or post-starburst nature, embedded in luminous disk galaxies and triggered by interactions or mergers, with only a minority of weak AGN. For $I \leq 20.5$ mag, these disk galaxies are seen out to a redshift $z \approx 0.4$, whereas at $I \approx 22.5$ mag, they are expected^{2,20,28} to be at $z \leq 0.8$. Higher resolution VLA B-configuration and observations with the refurbished HST will help locate any weak AGN, as these

may occur together with nuclear and/or global starburst in the microjansky radio sources, possibly triggered by the same dynamical disturbance. □

Received 2 March; accepted 2 May 1995.

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ACKNOWLEDGEMENTS. Some observations reported here were made by The NASA/ESA Hubble Space Telescope, obtained at the Space Telescope Science Institute (STScI), which is operated by the Association of Universities for Research in Astronomy, Inc., under contract to NASA. Some optical observations were obtained at the Multiple Mirror Telescope Observatory, a joint facility of the University of Arizona and the Smithsonian Institution. Some observations were obtained at the National Radio Astronomy Observatory, which is operated by Associated Universities Inc., under cooperative agreement with the US NSF. We thank the VLA and STScI staff for their continuous help, and E. Wyckoff for help with the WF/PC image stacking. This work was supported by two NASA/HST grants from STScI, and by the US NSF.

Extreme elongation of asteroid 1620 Geographos from radar images

S. J. Ostro*, K. D. Rosema*, R. S. Hudson†, R. F. Jurgens*, J. D. Giorgini*, R. Winkler*, D. K. Yeomans*, D. Choate*, R. Rose*, M. A. Slade*, S. D. Howard* & D. L. Mitchell*

* Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91109-8099, USA

† School of Electrical Engineering and Computer Science, Washington State University, Pullman, Washington 99164-2752, USA

MOST small asteroids are thought to result from catastrophic collisions¹, and their shapes can provide insight into their origin and collisional evolution. Two main-belt asteroids have been successfully imaged by spacecraft^{2,3}, but such images have yet to be obtained for asteroids that cross Earth's orbit. Earth-crossing asteroids are generally too small to be resolved by optical telescopes, and shape constraints derived from optical lightcurves are subject to large systematic biases⁴. Ground-based radar observations, on the other hand, have proved successful in resolving the

TABLE 1 Observation parameters

Date	Runs	UTC interval	Phase interval
30 Aug.	3	05:15–05:20	310°–315°
	43	05:23–07:07	320°–64°
	50	07:13–09:02	86°–211°
	27	09:07–10:01	218°–279°
31 Aug.	21	03:39–04:30	59°–120°
	52	05:43–07:28	202°–322°
	54	07:38–09:27	335°–99°

Columns give date in August 1994, the number of runs, the UTC time interval, and the rotation-phase interval for each sequence of 0.5- μ s imaging runs. Breaks between sequences were either caused by equipment problems or introduced to allow data transfer between acquisition and analysis computers. Geographos's topocentric right ascension, declination and distance near the middle of the observations on 30 and 31 August were (321.6° and 321.7°, respectively), (–23° and –18°) and (0.048 and 0.053 au). All observations employed transmission of a circularly polarized signal and reception of echoes in the orthogonal circular polarization. We used a repetitive, binary-phase-coded waveform³⁸ with a 127-element code that provided 127 'range bins', each with a time resolution, 0.5 μ s, equal to the temporal extent of each code element. Application of a 64-point fast Fourier transform to successive, decoded voltage samples from a single range bin sorted those echoes into 64 frequency bins³⁹. Each delay-Doppler image was constructed by repeating this process for each range bin.

shapes of some small asteroids^{5–9}. We describe here radar measurements of the Earth-crossing asteroid 1620 Geographos during its recent close encounter with the Earth. We have determined the silhouette of Geographos along its rotation axis, and confirm earlier lightcurve-based conjectures¹⁰ that this object has a very unusual shape. The silhouette is irregular, non-convex and has an aspect ratio of 2.76 ± 0.21 , establishing it as the most elongated Solar System object yet imaged. The unusual nature of the shape is underscored by laboratory fragmentation experiments^{11,12}, in which the average aspect ratio of fragments is 1.4, with fewer than 1% as elongated as Geographos.

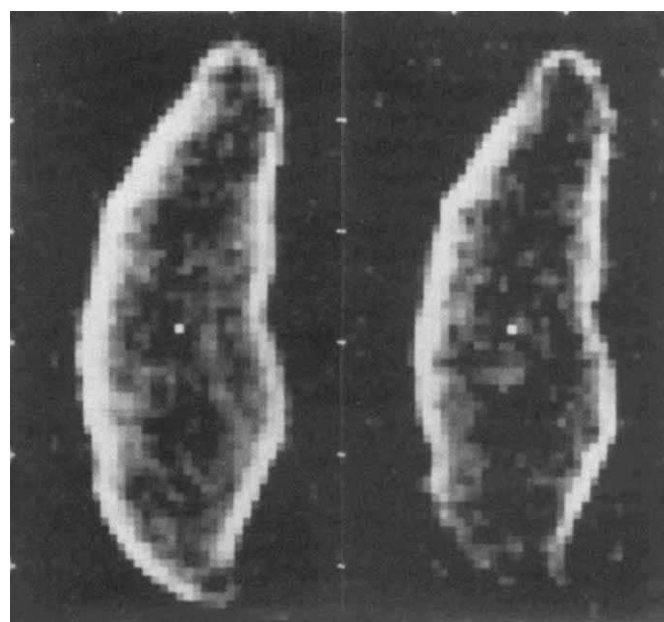
The Earth-crossing Apollo asteroid 1620 Geographos was discovered at Palomar Observatory on 14 September 1951, by A. G. Wilson and R. Minkowski. Geographos approached to within 0.061 au of Earth on 26 August 1969, and extensive photometric and polarimetric observations by Dunlap¹⁰ during January, August, September and October of that year yielded lightcurves whose enormous brightness oscillations ($B_{\max}/B_{\min}$ as large as 6.5) exceeded those observed for any other asteroid, a distinction that still holds. The 1969 results prompted speculations about a "cigar-shaped" object and inspired a painting by *National Geographic* artist D. Meltzer (p. 175 of ref. 13) that also forms the frontispiece for the first modern scientific book on asteroids¹⁴.

In August 1994, Geographos passed within 0.034 au of Earth, its closest approach for at least the next two centuries. The asteroid entered the declination window of the Goldstone radar on 28 August and we observed it daily for one week, obtaining hundreds of images. Here we present the first results from our highest-resolution observations.

Each of our images shows the distribution of echo power in time delay (range) and Doppler frequency (radial velocity). Contours of constant delay are defined by planes normal to the line of sight, and for a rotating rigid body, contours of constant frequency are defined by planes parallel to both the line of sight and the target's apparent spin vector. Thus, each image cuts the target into cells that are parallel to the apparent spin vector's plane-of-sky projection¹⁵. Constraints on Geographos's pole direction from optical lightcurves^{10,16} indicate that Goldstone was within 10° of the asteroid's equatorial plane throughout the radar observations, so the delay-Doppler cells remain nearly normal to the asteroid's equatorial plane at all rotation phases. Because each cell can capture echoes from surface regions north and south of the equator, an image is a 'double exposure' that

contains an intrinsic north/south ambiguity. However, sums of co-registered images over a sufficient range of phases can unambiguously define the asteroid's pole-on silhouette, that is, the outline of the object when viewed from along the pole.

Our highest-resolution observations consisted of 250 runs on 30–31 August (Table 1). In each run, Goldstone's 70-m antenna (DSS 14) transmitted a 450-kW signal for ~50 s and then received echoes for a somewhat shorter duration. The combination of the transmitted waveform's modulation and our real-time processing of received signals provided a time resolution of 0.5 μ s (75 m in range) and a frequency resolution of 1.64 Hz (2.9 cm s^{–1} in radial velocity). The length equivalent (87 m) of the frequency resolution and our rotation-phase assignments were calculated from a topocentric ephemeris and comprehensive, lightcurve-based estimates (P. Magnusson, personal communication) of the spin period ($5.22332784 \pm 0.00000096$ h) and the pole direction (ecliptic longitude, latitude is $55^\circ \pm 5^\circ$,



30 August

31 August

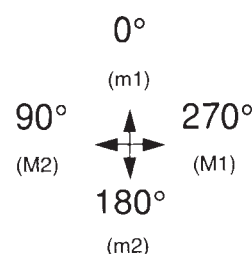


FIG. 1 Estimates of Geographos's pole-on silhouette from co-registration and summation of all 0.5- μ s images obtained on 30 and 31 August 1994. The salient information in this image is the periphery of the echo distribution; the grey-scale is arbitrary and no meaning is attached to brightness variations inside the silhouette. The tick marks on the borders are 1 km apart. The central white pixel locates the asteroid's centre of mass. Our rotation-phase origin (0° in the diagram below the images) corresponds to the asteroid's end-on orientation near primary (dimmiest) lightcurve minimum m1, which follows primary (brightest) lightcurve maximum M1. Geographos's lightcurves show extrema in the chronological order m1, M2, m2, M1, corresponding to phases near 0°, 90°, 180° and 270°. Arrows indicate the direction to the radar at each of those phases.

$-45^\circ \pm 5^\circ$); relative errors in our phase assignments are thought to be very small compared to the $\sim 0.6^\circ$ phase resolution of each run.

The extremely high noise level in images from individual runs is reduced by an order of magnitude when all the runs from a given date are summed. Our precise knowledge of the spin period makes rotational co-registration of images straightforward. Of more concern is "translational smearing" caused by imperfect knowledge of the delay-Doppler location of the centre of mass from image to image. Our prediction ephemerides were accurate enough to prevent perceptible smearing over timescales of the order of 10 min, but much longer summations require an *a posteriori* ephemeris that is at least two orders of magnitude more accurate. Preliminary application of several analysis methods^{9,17} to low-resolution data from 28 August to 2 September has produced an ephemeris for which the centre-of-mass trajectory during 30–31 August is believed to contain uncertainties that degrade the data's intrinsic resolution by no more than several tens of per cent on each day.

Figure 1 shows estimates of Geographos's pole-on silhouette from co-registration and summation of single-run images. Although the echoes were stronger and the phase coverage was better on 30 August, the two estimates are in excellent agreement. Our estimates of the silhouette's extreme breadths (that is, the extreme dimensions of its convex envelope) are 5.11 ± 0.15 km and 1.85 ± 0.15 km; the stated uncertainties are subjective standard errors. Our estimate of the asteroid's elongation (that is, the ratio of the silhouette's extreme breadths) is $\beta = 2.76 \pm 0.21$; we have assumed a cross-correlation coefficient¹⁸ of 0.5 between uncertainties in the breadth estimates, because they suffer similar biases from errors in the *a posteriori* ephemeris. These results show that lightcurve-based predictions about Geographos's elongation were accurate. For example, Dunlap's¹⁰ 'best fitting model (a cylinder with hemispherical ends) had a length to width ratio of 2.7, which results in Geographos being 1.50 ± 0.15 km wide and 4.0 ± 0.5 km long', and Kwiatkowski¹⁶ reported an ellipsoidal model with $\beta = 2.5 \pm 0.3$. The most exhaustive study of Geographos lightcurves to date yields an ellipsoidal model with $\beta = 2.58 \pm 0.16$ (ref. 19). Our results also support the general conclusion (resulting from the Galileo spacecraft flybys of the much larger, main-belt asteroids 951 Gaspra and 243 Ida) that shape models based on well determined lightcurves are reliable predictors of those objects' elongations^{2,3,20,21}.

How rare is Geographos's elongation among the Earth-crossing asteroids (ECAs) and main-belt asteroids (MBAs) of similar size? Optical lightcurves are available for dozens of ECAs and small MBAs, and a lightcurve's peak-to-valley amplitude, that is, the ratio of the maximum and minimum brightnesses $B_{\max}/B_{\min}$, is often taken as a crude indicator of elongation despite the potentially strong dependence of $B_{\max}/B_{\min}$ on viewing/illumination geometry⁴. Binzel *et al.*²² noted that whereas amplitudes taken at face value implied mean elongations of ~ 1.6 for 32 ECAs and ~ 1.3 for 32 small MBAs, mean elongations between 1.2 and 1.3 were inferred for both groups if 'global' corrections were applied to the amplitudes to compensate for primary sources of bias. Radar delay-Doppler and Doppler-only images have yielded direct determinations of β for the Mars-crossing asteroid 433 Eros²³ ($\beta \approx 2.1$) and about a dozen ECAs, including 1627 Ivar⁶ ($\beta \approx 2.1$), and 4769 Castalia⁹ ($\beta \approx 1.6$). Those asteroids, as well as Gaspra²⁴ ($\beta \approx 1.7$), Ida^{3,25} ($2.23 \leq \beta \leq 2.39$), and all other Solar System objects for which β has been measured from optical images^{26,27}, are less elongated than Geographos.

What can be said about the detailed sequence of collisional events that produced Geographos's remarkable elongation? Current understanding of collisional processes rests largely on hyper-velocity fragmentation experiments and theoretical scaling laws^{11,12,28}. Laboratory experiments have involved a wide range of impact velocities and target/projectile materials^{29–31}, includ-

ing weakly bonded aggregates of stronger constituent particles³². Mean fragment elongations from experiments reported so far are typically ~ 1.4 , with fewer than 1% as elongated as Geographos. For experiments that used open-air detonation of spherical targets by explosive contact charges³³, mean fragment elongations are ~ 1.7 , with several per cent more elongated than Geographos. Breakage of fragments against chamber walls in experiments not carried out in the open may or may not bias results toward smaller elongations. On the other hand, small asteroids are thought to be the product of several generations of disruptive collisions¹, so one might argue that experiments involving secondary fragmentation against chamber walls provide the more relevant results. Capaccioni *et al.*³⁴ noted a tendency for fragments generated farther from the impact point to become larger and possibly more irregular in shape, and Paolicchi *et al.*³⁵ suggested that elongated fragments may be more likely if the collision-induced velocity field is highly asymmetric, but other clues about how elongated fragments might form are lacking. Furthermore, the degree to which laboratory results can accurately be extrapolated to asteroidal scales may be rather limited²⁸.

Apart from its gross dimensions, one of the more interesting attributes of Geographos's silhouette is the disparity between the contours of its two long sides. The middle of the M1 side (Fig. 1) contains a prominent indentation, but the entire length of the opposite (M2) side is nearly convex at the 100-m scale. To our eyes, the silhouette's shape seems more suggestive of a monolithic fragment derived from a disruptive collision than a compound, multi-component product of a constructive collision. However, our data hardly rule out the latter possibility. Moreover, if Geographos were an agglomeration of disparate fragments³⁶, clues about this configuration might not be evident from the silhouette. A three-dimensional model of Geographos could prove more informative, but due to the equatorial view of our observations, inversion of the images using the technique applied successfully to Castalia⁹ cannot produce a unique three-dimensional model of Geographos. Inclusion of lightcurves in the inversion may provide the additional geometrical information required for such a reconstruction³⁷. □

Received 24 March; accepted 17 May 1995.

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ACKNOWLEDGEMENTS. We thank the Goldstone technical staff for their support of these observations. Part of this research was conducted at the Jet Propulsion Laboratory, California Institute of Technology, under contract with NASA.

Ice in the 1994 Rabaul eruption cloud: implications for volcano hazard and atmospheric effects

W. I. Rose*, D. J. Delene*, D. J. Schneider*,
 G. J. S. Bluth*, A. J. Krueger†, I. Sprod†,
 C. McKee‡, H. L. Davies§ & G. G. J. Ernst||

* Geological Engineering and Sciences,
 Michigan Technological University, Houghton, Michigan 49931, USA
 † NASA Goddard Space Flight Center, Greenbelt, Maryland 20771,
 USA

‡ Rabaul Volcano Observatory, PO Box 386, Rabaul,
 Papua New Guinea

§ University of Papua New Guinea, PO Box 414, University,
 Papua New Guinea

|| Department of Geology, University of Bristol, Bristol BS8 1RJ, UK

VOLCANIC clouds are an important natural hazard to aircraft¹, and host chemical reactions that interest both volcanologists^{2,3} and atmospheric scientists^{4–6}. Ice has been suggested as a possible component of eruption clouds⁷, but there has been no direct evidence for its presence. Here we report the detection, using a satellite-borne infrared sensor, of ≥ 2 million tonnes of ice in the cloud produced by the September 1994 eruption of Rabaul volcano, in Papua New Guinea. The cloud also contained relatively low levels of sulphur dioxide (80 ± 50 kilotonnes), compared with other stratospheric eruption clouds. The unusual aspects of this cloud may be related to the entry of sea water into the volcanic vent, and its participation in the eruption column. Past eruptions that occurred in similar (coastal) settings, such as those of Krakatau and Santorini, might have had less effect on the atmosphere than their volume alone would suggest, because the presence of ice may decrease the residence time of ash and sulphur in the atmosphere. In addition, the ability of ice to mask the characteristic spectral signature of volcanic ash will increase the difficulty of designing airborne ash detection systems for aviation safety.

The 1994 Rabaul eruption began on 19 September with nearly simultaneous outbursts from two vents (Tavurvur, 06:06 local time; Vulcan, 07:17 local time) on opposite sides of the caldera (Fig. 1 inset). The Vulcan eruption was more powerful, with column heights estimated at 20 km, whereas Tavurvur's column reached a maximum height⁸ of 6 km. Some of the ash fallout was very wet, and a 'rain of mud' occurred in some areas around Rabaul. Sea water had access to the main active vent low on the northeastern flank of Vulcan, and salty rainfalls took place in a wide arc north and northwest of Vulcan during 19 and 20 September⁸. The tephra fall deposits resulting from Vulcan's eruptions contained ubiquitous sea-salt deposits.

The advanced very-high-resolution radiometer (AVHRR) detector aboard the NOAA 12 polar-orbiting satellite was used for mapping the volcanic cloud and determining the composition, size and mass of its particles based on data collected on 19 September at 09:00 UT (19:00 local time) and again at 21:50 UT (20 September, 07:50 local time). The dispersal pattern shown by the Rabaul cloud was marked by a broad fan shape (Fig. 2),

probably caused in part by upper-level winds; many volcanic clouds, particularly at middle and high latitudes, are more directionally focused⁹ than this.

Multi-wavelength AVHRR images have two thermal infrared channels (band 4, $\lambda = 10.3\text{--}11.3\ \mu\text{m}$; band 5, $\lambda = 11.5\text{--}12.5\ \mu\text{m}$) which can be used for estimation of the sizes, masses and some compositional characteristics of particles in the cloud¹⁰. Silicate ash and concentrated sulphuric acid aerosol particles in transparent drifting volcanic clouds have distinctive negative band 4–band 5 apparent temperature differences^{11–14}. Figure 3a shows a plot of band 4–band 5 temperature differences versus band 4 temperatures for the individual pixels comprising Fig. 2. For comparison, Fig. 3b shows a similar plot for the volcanic cloud arising from the Klyuchevskoi eruption. The Klyuchevskoi cloud is representative of many volcanic clouds which have negative band 4–band 5 temperature differences. The positive band 4–5 temperature difference exhibited by the Rabaul cloud is unique among a dozen eruptions that we have studied with this method¹³; the volcanic cloud shows no silicate or sulphate signal. The observed temperature differences were compared with simulated temperature differences calculated using refractive indices for ice (Fig. 3a), which demonstrates that Rabaul's cloud had the spectral characteristics of spherical ice particles with effective radii of 9–40 μm . (In reality, the cloud will have contained particles with radii outside this range.) Note that the temperature of the opaque portion of the Rabaul eruption cloud is $\sim 190\ \text{K}$ ($-83\ ^\circ\text{C}$), corresponding to the inferred tropopause temperature from the radiosonde data.

We compare Rabaul to the Klyuchevskoi eruption because the Klyuchevskoi cloud is much more like other volcanic clouds we have studied, was similar in scale to Rabaul, although smaller, and was examined by the same AVHRR detector only 12 days after it observed Rabaul's cloud. The Klyuchevskoi cloud contained more SO_2 than Rabaul's, although little ice, and was driven by stronger ($40\text{--}65\ \text{m s}^{-1}$) northwesterly winds at an altitude of 12–13 km. The Klyuchevskoi cloud exhibited no significant wind shear (P. Newman, personal communication) and a focused dispersal pattern which extended in an elongated plume across the northern Pacific. The effective radii of the ice particles in the Rabaul cloud are larger than the particle sizes that we have observed in volcanic clouds with silicate signals, such as Klyuchevskoi. The effective particle radius inferred for the Klyuchevskoi eruption cloud was in the range 1–12 μm .

Using the methods of Wen and Rose¹⁰, we computed the total masses of ice in the transparent (optical depth < 4) parts of the Rabaul cloud and found a mass of 2–3 megatonnes (Mt). This mass is ten times the mass of silicates calculated for the Klyuchevskoi cloud (0.1–0.3 Mt). The particle mass contained in the transparent part of a volcanic cloud during an eruption is a small fraction ($< 1\%$) of the total¹⁰, as most of the mass is in the opaque near-vent portion of the cloud. The opaque por-

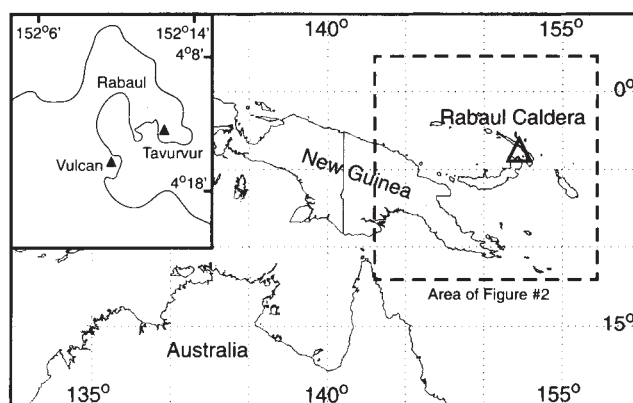


FIG. 1 Location maps for Rabaul caldera.

tion of Rabaul's cloud (optical depth >4) makes up $<5\%$ of the total area (Fig. 2) but must contain all of the large particles (>1 mm in diameter), which fall out in less than 30 minutes before they travel more than a few tens of kilometres. Smaller particles fall out more slowly but represent a small fraction of the total particle mass in the cloud. For instance, a 13-hour-old drifting cloud from the September 1992 Spurr eruption contained a mass equivalent to $<1\%$ of the mass that fell out in the ash-fall blanket¹⁰. We cannot measure the masses in the dense opaque portion of Rabaul's cloud near the vent, where fallout is highly concentrated and particles are largest, but based on analogy with the Spurr cloud, we conclude that the total ice mass in the Rabaul cloud is likely to be at least 100 times higher than our estimate for the transparent cloud.

The Total Ozone Mapping Spectrometer (TOMS) aboard the Meteor 3 satellite was used to map and quantify the SO_2 mass in the Rabaul cloud¹⁵. The TOMS detected SO_2 on five days (19–23 September), obtaining daily values of 30–80 kt and a total five day mass estimated at 200 ± 100 kt. The determination of 80 ± 50 kt SO_2 in the cloud on 20 September was most nearly simultaneous with our AVHRR measurement. Following the eruption, correlation spectrometer measurements of SO_2 emissions from Tavorvur declined from 29 September to 6 October, from ~ 30 to less than 3 kt d^{-1} (S. N. Williams and S. I. Schaefer, personal communication).

We interpret the observations described above as reflecting the influence of sea water in the Vulcan vent (which is close to sea level) and its incorporation into the eruption column. We

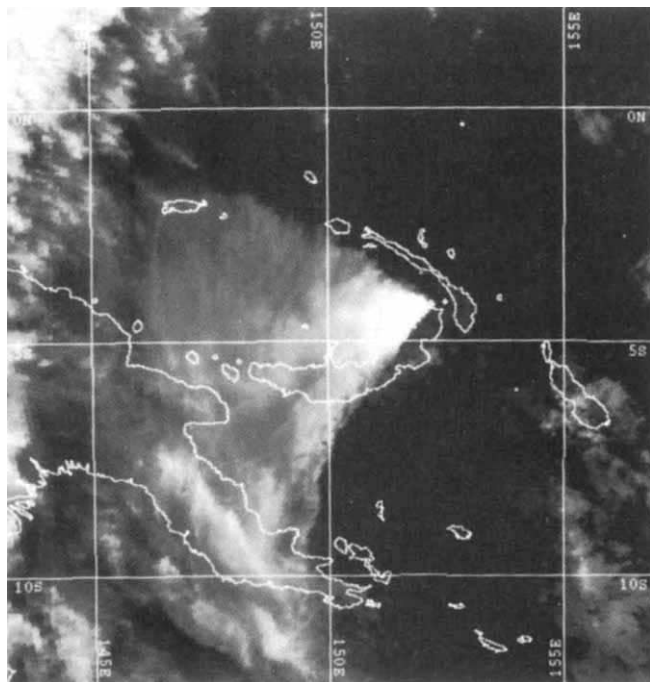
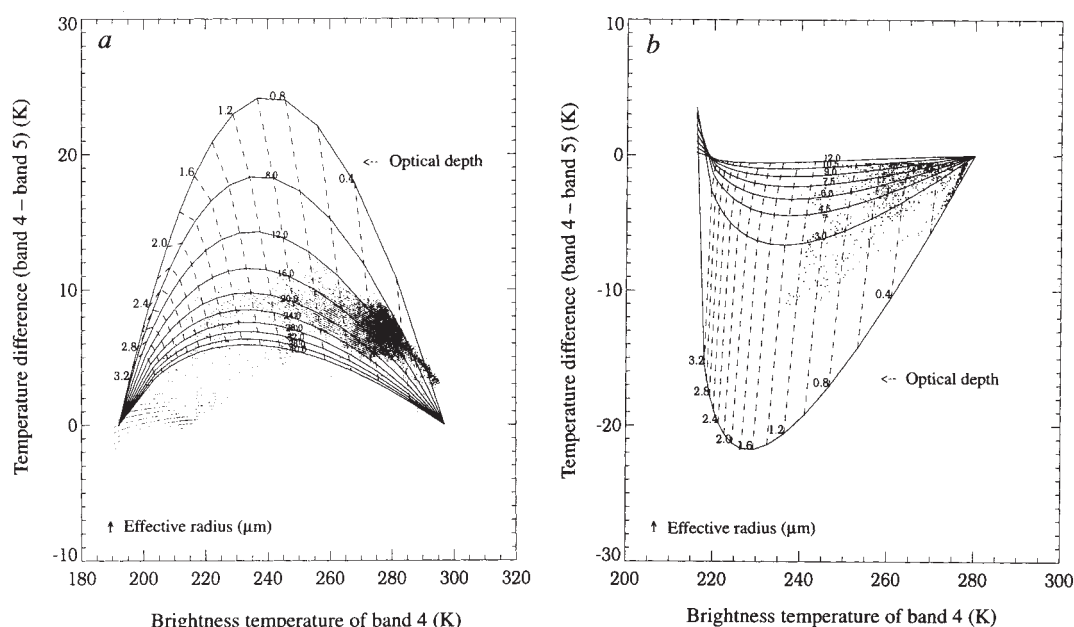


FIG. 2 AVHRR image of the Rabaul eruption cloud at 09:00 UT, 19 September 1994, showing band 4 data with the coldest temperatures shown by the brightest pixels. The temperature of the opaque part of the Rabaul cloud (optical depth >4) is ~ 190 K. Most of the cloud is transparent, and the warmer surface below transmits through the cold cloud, resulting in higher measured temperatures. The transparent part of this cloud is used for the plot of individual pixel values in Fig. 3a. High-level winds and temperature profiles were obtained through NASA's Goddard Space Flight Center from several radiosonde stations surrounding Rabaul for 18 and 19 September at 23:00 UT each day (the closest being the station at Momote, ~ 540 km WNW of Rabaul). They show the tropopause at an altitude of 16 km, with a temperature of 190 K. The prevailing winds exhibit wind shear, with generally stronger (initially 25–35 and increasing to 50–60 m s^{-1}) northeasterly winds at 10–13 km height and weaker (15–45 m s^{-1}) ESE winds at 16–21 km (P. Newman, personal communication).

FIG. 3 Band 4 – band 5 temperature differences plotted versus band 4 brightness temperatures from NOAA 12 AVHRR images. Points correspond to individual pixels in the volcanic clouds. *a*, The cloud from the Rabaul eruption, at 09:00 UT on 19 September 1994, shows marked positive band 4 – band 5 temperature differences. *b*, The cloud from the Klyuchevskoi eruption, at 06:30 UT on 1 October 1994, shows negative band 4 – band 5 temperature differences more typical of volcanic clouds. The simulated temperature results (lines in the figure) are compared with actual pixel values (points in the figure) for both eruptions, but for Klyuchevskoi we compare theoretical volcanic ash, based on refractive index data and lognormal size distributions following the method of Wen and Rose¹⁰. For the Rabaul data we follow an analogous approach using refractive-index data for ice and a



lognormal size distribution. For both figures the measured values of optical depth are plotted as dashed near-vertical lines, and effective radius is plotted as arcuate solid lines.

suggest that disturbance of the region around the vent resulted in ready access for sea water to the vent, and that once the sea water came in contact with the magma, it was vaporized and added to the convecting eruption column. Water droplets and ash particles interacted in the lower column, resulting in the evaporative formation of salt crystals¹⁶. The convecting column with its high content of water was cooled as the column rose to 20 km. The ash surfaces in the eruption column continued to nucleate liquid water and ultimately formed ice at temperatures below -40°C (refs 17, 18). The dominance of the ice signal in the AVHRR satellite data is explained by ash being encased within ice in this cloud. The formation of ice in volcanic clouds probably accelerates ash fallout by increasing the diameter of cloud particles³.

We speculate that SO_2 concentrations in the cloud are limited by the accumulation of water droplets, supercooled water and eventually ice around ash nuclei⁴. The mass of SO_2 dissolved in supercooled water (and subsequently trapped in ice) can be estimated at 20–100 kt using the methods of Tabazadeh and Turco⁶ for a volcanic cloud at an altitude of 14 km with a total ice mass of 200–300 Mt (based on the data discussed above). The participation of sea water in the eruption column may thus account for the low amounts of SO_2 detected by TOMS in the case of Rabaul's eruption. We note for example, that in the case of Mount St Helens in 1980 the rates of SO_2 release measured during the climactic eruption were orders of magnitude higher than the rates measured after the eruption, during an open vent condition¹⁹. In contrast, the measurements by TOMS at Rabaul on 29 September (from Tavurvur) were almost the same as those detected during the main Vulcan eruption. In the record of TOMS data since 1979, most stratospheric eruptions, even 'sulphur-poor' ones, have released at least several hundred kilotonnes of SO_2 (ref. 20). These data are consistent with the speculation that much more SO_2 was released during the Rabaul eruption, but that it was scavenged by the H_2O -rich fallout. We speculate that past eruptions with similar sea-level or lake locations (for example, Krakatau²¹, Taupo²² and Santorini²³) could also have been influenced by ice formation, which reduced the length and severity of their influence on the atmosphere by enhancing the removal of both sulphur and ash. We also speculate that the broad 'fan-like' dispersion pattern of the cloud could partly reflect seawater interaction, inducing vorticity by evaporation of water droplets at the cloud edges^{24,25}.

Our results demonstrate the utility of multi-wavelength remote sensing in identifying the constituents of volcanic clouds. Our observations are also relevant to the problem of mitigating the hazard posed to jet aircraft by volcanic clouds that contain silicates which can damage and stop their engines¹. Although they do not display the radiative signal of silicate particles, clouds like Rabaul's may still be hazardous to aircraft, because although ice makes up much of the mass of the cloud, the particles probably have silicate cores. The design of airborne devices to discriminate between water/ice clouds and volcanic ash clouds for aircraft hazard mitigation purposes²⁶ should try to address the eventuality posed by the Rabaul example. We note that there are many Western Pacific and Indonesian volcanoes near important air routes and with settings similar to Rabaul²⁷, and many of these volcanoes have drifting cloud patterns that either disperse in broad fans²⁸ or bifurcate²⁴. □

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ACKNOWLEDGEMENTS. We acknowledge conversations with T. Gerlach, R. Turco, P. Hobbs and S. Williams, and thank P. Newman for giving us access to radiosonde data, W. Johnson for help with communications and J. Crisp for reading the rough draft and helping to improve it. G.G.J.E. acknowledges R. S. J. Sparks, J. P. Davis, P. Leonard, F. Wheeler, W. Bowery for collaborative support and inspiration, a NERC Assistantship (BRIDGE program) and the 1994 award from the Fondation Belge de la Vocation. W.I.R., D.J.D., D.J.S. and G.J.S.B. were supported by NASA through the Volcano/Climate program.

Granulite formation during continental extension in Fiordland, New Zealand

G. M. Gibson* & T. R. Ireland†

* University of Southern Queensland, Toowoomba, Australia 4350

† Australian National University, Canberra, Australia 0200

THE formation of the high-pressure, high-temperature rocks known as granulites has traditionally been attributed to metamorphism in the deeper parts of continental collision zones and magmatic arcs^{1–3}. More recently, greater consideration has been given to the possibility of granulite formation during continental rifting^{4–6}, and in particular⁴ to whether granulites are forming today beneath the North American Basin and Range province. Here we report thermobarometric data and single-zircon U–Pb ages for an analogous, but much more deeply exhumed, extensional terrain in Fiordland, New Zealand^{7,8}, where Early Cretaceous granulites underwent renewed granulite-facies metamorphism at significantly shallower crustal levels following the extensional collapse of magmatically thickened continental crust. Our data show that granulite-facies metamorphism can take place during continental extension, and demonstrate that in western New Zealand it was accompanied at higher structural levels by the emplacement of one or more metamorphic core complexes^{7–9}, in keeping with the model postulated⁴ for the Basin and Range province.

Granulites in Fiordland (Fig. 1) were derived from Early Cretaceous plutonic protoliths of intermediate to basic composition^{7,10–12} and include both garnet and two-pyroxene varieties. They make up the regionally extensive Western Fiordland Orthogneiss (WFO) and have undergone polyphase metamorphism (M1–M3) and at least three phases of deformation (D1–D3), the youngest of which deforms the WFO into a series of open, post-metamorphic folds^{8,12,13}. These folds are of probable late Tertiary age and were superimposed on an earlier (D2) streaky to well developed gneissic foliation that is in part mylonitic. Mylonitic fabrics were originally subhorizontal and occur in both preserved granulites and those subsequently retrogressed to the amphibolite facies; they reflect deformation under progressively changing metamorphic conditions as the WFO was uplifted to shallower crustal levels.

Markedly different metamorphic pressures are obtained for garnet granulite assemblages that predate (M1) and postdate

Received 16 February 1995; accepted 10 May 1995.

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TABLE 1 Thermobarometric determinations for coexisting garnet–clinopyroxene pairs in Western Fiordland Orthogneiss

Sample	X_{Ca}^{gt}	X_{Fe}^{gt}	X_{Mg}^{gt}	X_{Fe}^{cpx}	X_{Mg}^{cpx}	T^{EG} (°C)	P^{NP} (kbar)	P^M (kbar)	P^{PH} (kbar)
M1 garnet granulite assemblages									
86-54C	0.1002	0.4002	0.4275	0.2141	0.7859	832	11.9	12.5	11.2
86-54R	0.1080	0.4675	0.3489	0.2117	0.7883	723	10.6	11.1	9.9
88-35C	0.1830	0.4237	0.3574	0.2037	0.7963	800	11.8	12.2	11.2
88-35R	0.1997	0.4249	0.3460	0.1909	0.8091	791	12.1	12.4	11.5
M2 garnet granulite assemblages									
86-52C	0.0634	0.4617	0.3611	0.2533	0.7467	766	6.5	6.1	4.5
86-52R	0.0860	0.4717	0.3409	0.2563	0.7437	768	7.8	8.2	6.9
88-39C	0.1424	0.4794	0.3315	0.2136	0.7864	734	9.8	10.3	9.1
88-39R	0.1391	0.4910	0.3262	0.2067	0.7933	709	9.7	10.2	9.0

Compositional data for garnet (gt) and clinopyroxene (cpx) have been derived from electron-microprobe analyses after adjustment for Fe^{3+} . Cation proportions (X) calculated according to standard procedures^{14,16}. X_{Ca} is X_{Ca} less the amount of Ca incorporated in the andradite component of garnet. T^{EG} is the temperature (± 50 °C) calculated from ref. 27. P^{NP} , P^M and P^{PH} are pressures (± 1.5 kbar) after refs 14, 16 and 28, respectively, using the GADS equilibrium (garnet, anorthite, diopside, quartz). In M2 assemblages garnet formed at the expense of plagioclase and clinopyroxene, typically occurring as small (0.15–2 mm) grains clustered around pyroxene aggregates elongated parallel to the regional stretching direction. These garnets, many with inclusions of vermicular quartz, originated through a reaction (clinopyroxene + plagioclase = garnet + quartz) commonly observed in granulite terrains that have undergone isobaric cooling^{5,6}. In contrast, M1 garnets are extensively fractured; they have been variably replaced by clinopyroxene and predate the D2 deformation and associated fabrics that wrap around them. In extreme cases M1 garnets have been reduced to small, isolated relicts within pyroxene. In the sample names, C indicates thermobarometry on paired mineral cores, and R indicates thermobarometry on mineral rims. All calculations are based on a minimum of five mineral pairs per sample. P^{NP} includes empirical adjustment (+1.6 kbar) recommended in ref. 14.

(M2) the onset of D2 deformation (Table 1). The former yield high pressures (10–12 kbar) consistent with a lower crustal origin, whereas M2 assemblages formed at lower pressure (5–10 kbar) and shallower crustal levels. High pressures have also been reported^{14–16} for subplanar zones of garnet granulite developed in and around anorthositic veins and dykes that cut across the mylonitic fabric but which have themselves been subsequently deformed and overprinted by later M2 garnet granulite assemblages. These veins and dykes were evidently intruded into the WFO during the earlier stages of D2 deformation whilst the latter was still at lower-crustal depths. Previously, all garnet granulite assemblages were thought^{10,11,17,18} to have originated during a single episode of metamorphic mineral growth.

Temperatures calculated (Table 1) for M2 metamorphism (710–780 °C) are not appreciably different from those obtaining during initial high-pressure metamorphism (800–830 °C). This suggests that the initial stages of D2 deformation were accompanied by near-isothermal uplift. Cooling obviously did not occur at lower-crustal levels but was delayed until the WFO was uplifted to much shallower levels. A metamorphic history involving near-isothermal decompression followed by cooling at mid-crustal levels is indicated for the WFO. Clockwise pressure–temperature–time paths such as this are considered¹⁹ rare for granulite terrains, but are consistent with theoretically derived metamorphic histories for granulite terrains that underwent extensional collapse following earlier crustal thickening^{5,6,20}.

With further uplift and cooling, metamorphic conditions in the WFO changed from the granulite to amphibolite facies (M3) and strain became increasingly partitioned into narrow shear zones from which all vestige of original granulite-facies meta-

morphism has been obliterated. Amphibolitized shear zones are of extensional origin and display a range of fabrics (S–C bands, rotated porphyroclasts) consistent with a normal sense of displacement^{7,8}. One such shear zone separates the WFO from the cover sequence^{12,13} (Fig. 1) and in Doubtful Sound has brought high-pressure (12 kbar) garnet granulites within 1 km of pelitic schists metamorphosed at 7–9 kbar; some 10–12 km of middle crust has been excised⁸. Moreover, this and other extensional shear zones in the region share the same sense of asymmetry as upper-crustal shear zones associated with emplacement of the Paparoa metamorphic core complex in the formerly contiguous Nelson–Westland region⁹ (Fig. 1). Stretching lineations and kinematic indicators in retrogressed WFO and the Paparoa core complex give a mainly top-to-the-northeast sense of shear^{8,9}. K–Ar and Rb–Sr mineral ages^{7,9,11,21} for both units are also very similar, indicating rapid cooling at all crustal levels by mid-Cretaceous time. D2 uplift of the WFO and emplacement of the Paparoa core complex were thus concomitant processes; both are related to continental extension and both resulted from the same tectonic processes that eventually led to continental rifting and breakup of the Pacific margin of Gondwana^{7,9,22}. However, whereas the Paparoa core complex was exposed by mid-Cretaceous time⁹, the WFO remained at depth (10–12 km) until exhumed by an entirely separate tectonic event related to transpression on the late Tertiary Alpine fault^{8,13}.

Ion-probe U–Pb data for four WFO samples are shown in Fig. 2 and a summary of the age data is presented in Table 2. Three samples (94-186, 87-69 and 87-32) have U and Th concentrations, and Th/U ratios, that are in the range normally

TABLE 2 U–Th–Pb measurements for Western Fiordland Orthogneiss zircon samples

Sample	U (p.p.m.)	Th (p.p.m.)	Th/U	No.	Age (Myr)	χ^2	($^{207}Pb/^{206}Pb$) _m
87-69	180–850	110–841	0.48–1.25	10/10	126 ± 3		
87-32	98–197	62–200	0.63–1.17	8/8	119 ± 5		
94-186	130–250	120–270	0.77–1.24	10/10	119 ± 42.0	0.3	<0.06
91-588	4.2–10.3	0.03–0.08	0.004–0.009	9/10	107.5 ± 2.8	1.8	0.12–0.23

Three zircon samples (94-186, 87-69, 87-32) have U and Th concentrations and Th/U ratios (0.5–1.5) typical of the Western Fiordland Orthogneiss, yielding mean ages (119–126 Myr) consistent with conventional isotopic dilution analyses¹⁰. Zircons from 91-588 have low U (≤ 10 p.p.m.) and extremely low Th (< 0.1 p.p.m.) and low Th/U (0.01) indicative of high-grade metamorphic zircon^{29,30}. The low U concentrations result in extremely low radiogenic Pb levels and the total Pb measured is only of the order of 0.2 p.p.m. For definitions of χ^2 and No., see Fig. 2 legend. Ages are derived from the intercept with concordia of a regression line through the data and forced through common Pb of Broken Hill Pb isotopic composition. The goodness-of-fit parameter to the regression lines through the data points is given by χ^2 and the number of points used in the regression is given by No. Range of uncorrected $^{207}Pb/^{206}Pb$ ratios is given by ($^{207}Pb/^{206}Pb$)_m. Data for samples 87-69 and 87-32 are from ref. 7.

expected for magmatic zircon. Their mean ages (119–126 Myr) confirm the Early Cretaceous age of the WFO protolith and are consistent with conventional U–Pb ages previously published¹⁰ for this unit. In contrast, zircon from sample 91-588 yields a significantly younger age of 107.5 ± 2.8 Myr. Moreover, all zircons from this sample (50 crystals examined; 10 analysed) are characterized by extremely low levels of U (≤ 10 p.p.m.) and especially Th (< 0.1 p.p.m.). Both the chemistry and the age indicate that the zircon in this sample is not inherited from the granulite protolith, but instead represents a new generation of zircon growth related to a second episode of granulite metamorphism. This sample derives from an enclave of high-pressure garnet granulite that underwent partial melting during or immediately preceding the initial stages of D2 deformation. It is migmatitic and incorporates millimetre- to centimetre-sized segregations of anorthositic material in which patches of original M1 garnet and pyroxene persist. D2 deformation, uplift of the WFO and consequent M2 granulite metamorphism are therefore all constrained to be younger than 107.5 ± 2.8 Myr. Whether partial melting was itself a response to decompression is not immediately clear, although it cannot be attributed to the effects of magmatic heating as the enclave is hosted by older WFO dated at 126 Myr (Table 2).

Previous best estimates for the onset of continental extension range from 105 to 110 Myr, based mainly on the age of sedimentation and coeval silicic volcanism associated with emplacement of the Paparoa metamorphic core complex^{9,21,22}. Recently, a 110 ± 2 Myr ion-probe zircon age has been reported²³ for a strongly mylonitized granite (Buckland Granite) in the lower plate of this same complex. Its age helps constrain the age of ductile deformation associated with the onset of continental extension. This age is also identical within error to that of partial melting at lower-crustal depths in the WFO (see above), thereby providing further evidence that D2 deformation and uplift in the WFO were linked at shallower levels to granite intrusion and emplacement of the Paparoa core complex. More importantly, in view of the shared deformational histories, emplacement of the Paparoa core complex must have been accompanied at depth by

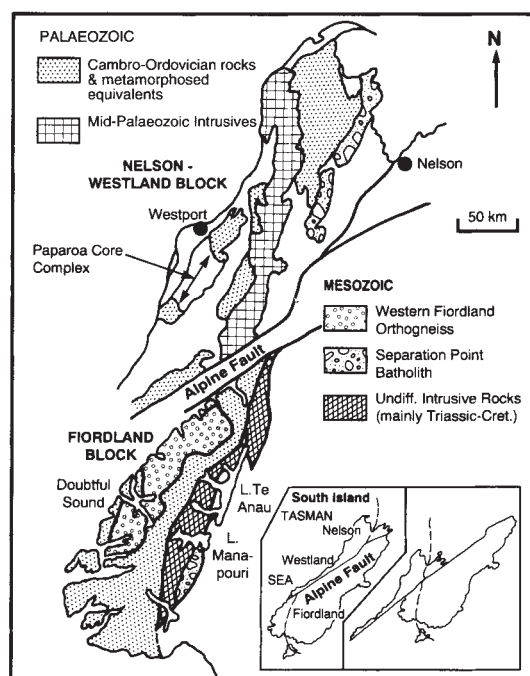


FIG. 1 Generalized geological map for western New Zealand showing distribution of rock units discussed in text. Insets show Fiordland and Nelson-Westland regions in their present and pre-Cenozoic configurations in relation to Alpine fault. After refs 9 and 10.

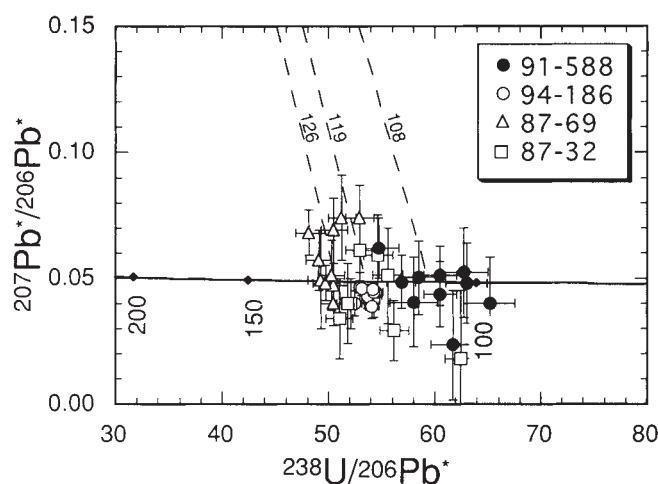


FIG. 2 Tera-Wasserburg concordia for four Western Fiordland Orthogneiss zircon samples. U/Pb ratios are normalized to a value of 0.0928 for the 572 MA SL13 standard. The data plotted are radiogenic Pb compositions as denoted by the asterisk. For samples 94-186 and 91-588, common lead has been estimated from the $^{208}\text{Pb}/^{206}\text{Pb}$ and Th/U ratios. For the other two samples (87-69, 87-32), common lead has been estimated from the $^{204}\text{Pb}/^{206}\text{Pb}$ ratio. The dashed lines illustrate mixing lines between common lead and radiogenic lead on concordia (solid line) of the ages shown. The data from 91-588 are clearly displaced from the main cluster of data for the other three samples. Error bars are 1σ uncertainty.

near-isothermal decompression and consequent M2 granulite-facies metamorphism. M2 garnet granulite assemblages postdate partial melting in the WFO and thus cannot be any older than 108 Myr. Further uplift of the WFO was accompanied by M3 amphibolite facies metamorphism and continued until at least the mid-Cretaceous by which time temperatures had fallen below 500°C as evidenced by a 93-Myr hornblende K–Ar age⁷ from a late D2 shear zone.

Continental extension and mid-Cretaceous granite plutonism were closely related in western New Zealand, mirroring the situation in the Basin and Range province, where silicic magmatism similarly attended the earlier stages of continental rifting²⁴. However, it is equally clear that this is not the only parallel between western New Zealand and the Basin and Range province. Both regions also share a similar history of crustal thinning, elevated heat flow and ductile deformation, culminating in the emplacement and unroofing of high-grade metamorphic core complexes. Above-average heat flow in the Basin and Range province has been cited⁴ as evidence for granulite formation at depth, and accords well with the analogous situation in western New Zealand where mid-Cretaceous heat flow must have been similarly high as evidenced by M2 granulite-facies metamorphism at mid-crustal levels. As in the Basin and Range province, exhumation in western New Zealand must have been sufficiently rapid to preclude significant metamorphic cooling until continental extension was essentially complete or at least well advanced.

Before continental rifting in late Mesozoic time, western New Zealand was the site of plate convergence and subduction-related arc magmatism, represented in Western Fiordland by the WFO and at higher structural levels in Nelson and Westland by the Separation Point batholith composed mainly of 114–125-Myr granitic plutons^{23,25}. However, unlike Fiordland, the Separation Point batholith is devoid of evidence for deep burial and Early Cretaceous high-pressure metamorphism. Crustal thickening in western New Zealand is thus more probably related to magmatic²⁶, rather than tectonic^{10,15,26}, processes. The WFO protoliths were intruded and initially metamorphosed at lower-crustal depths in an Andean-style tectonic environment. But by 110 Myr plate convergence and subduction-related magmatism had ceased or appreciably slowed, leading to extensional collapse

of the magmatically thickened, and already thermally weakened, continental arc. With the onset of continental rifting and related uplift, further granulite-facies metamorphism occurred at lower pressures. Previously, all garnet-granulite-facies metamorphism in Fiordland has been attributed to crustal thickening following an Early Cretaceous collision event¹⁵. Our data indicate that a clockwise rather than an anticlockwise pressure-temperature-time path is more appropriate for the granulites of Fiordland, and in this respect they might serve as lower-crustal analogues not only for the Basin and Range province but for other, much older extensional terrains where only the deeper crustal levels are now preserved. □

Received 5 December 1994; accepted 2 May 1995.

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ACKNOWLEDGEMENTS. For access to the ion-probe facilities at the Australian National University we thank W. Compston and D. H. Green; for reviews of the manuscript we thank R. J. Norris & G. J. H. Oliver. Garnet activities for the *P-T* determinations were calculated using a software package provided by P. J. O'Brien. Accommodation and access to a boat in Doubtful Sound were kindly provided by the Deep Cove Educational Trust. Permission to collect rock samples in Fiordland National Park was granted by the New Zealand Department of Conservation. This research was supported by the Australian Research Council.

Phantom-limb pain as a perceptual correlate of cortical reorganization following arm amputation

H. Flor*, T. Elbert†, S. Knecht‡, C. Wienbruch‡, C. Pantev‡, N. Birbaumer§, W. Larbig§ & E. Taub||

* Department of Psychology, Humboldt-University, Hausvogteiplatz 5–7, D-10117 Berlin, Germany

† Department of Psychology, University of Konstanz, Postfach 5560, D-44, D-78434 Konstanz, Germany

‡ Department of Neurology and Institute of Experimental Audiology, University of Münster, Kardinal-von-Galen-Ring 10, D-48129 Münster, Germany

§ Institute of Medical Psychology and Behavioural Neurobiology, University of Tübingen, Gartenstr. 29, D-72074 Tübingen, Germany

|| Department of Psychology, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

ALTHOUGH phantom-limb pain is a frequent consequence of the amputation of an extremity, little is known about its origin^{1–4}. On the basis of the demonstration of substantial plasticity of the somatosensory cortex after amputation⁵ or somatosensory deafferentation in adult monkeys⁶, it has been suggested that cortical reorganization could account for some non-painful phantom-limb phenomena in amputees and that cortical reorganization has an adaptive (that is, pain-preventing) function^{2,5,7,8}. Theoretical and empirical work on chronic back pain^{9,10} has revealed a positive relationship between the amount of cortical alteration and the magnitude of pain, so we predicted that cortical reorganization and phantom-limb pain should be positively related. Using non-invasive neuromagnetic imaging techniques to determine cortical reorganization in humans^{11–13}, we report a very strong direct relationship ($r=0.93$) between the amount of cortical reorganization and the magnitude of phantom limb pain (but not non-painful phantom phenomena) experienced after arm amputation. These data indicate that phantom-limb pain is related to, and may be a consequence of, plastic changes in primary somatosensory cortex.

A brief telephone interview was used to obtain information about the amount of phantom-limb pain in 65 upper-limb ampu-

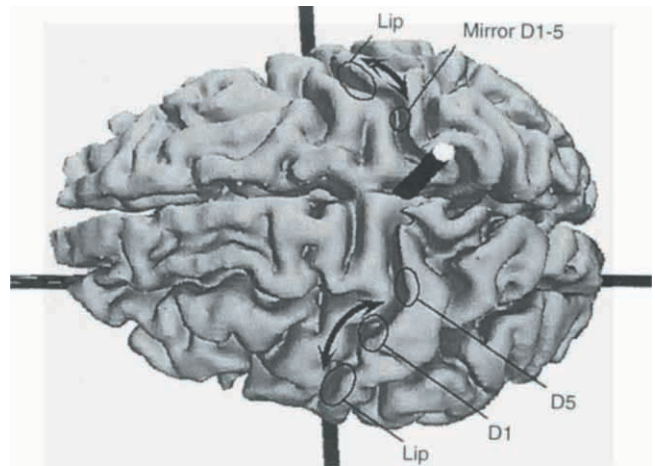
tees. This information served as the sole basis for the selection of a representative sample of 13 subjects with widely varying degrees of phantom-limb pain. The mean age of the 13 subjects was 50.1 years (s.d. = 17.2, range 27–73 yr), mean post-amputation time was 24.3 years (s.d. = 19.8, range 1 to 51 yr). Twelve men and one woman participated in the study. Traumatic injury in ten cases and osteosarcoma in three cases had made the amputation necessary. Cortical reorganization was determined by magnetic source imaging¹¹ using the method illustrated in Fig. 1. The subjects underwent a comprehensive neurological and psychological investigation which included detailed assessments of phantom pain and phantom sensations, stump pain and stump sensations, pre-amputation pain, telescoping (the subjective experience of the phantom limb retracting towards and often disappearing in the stump), and facial remapping (the appearance of phantom sensations upon non-painful stimulation of the face with isomorphism between facial stimulation sites and the location of phantom sensations) (Fig. 2 legend).

A large significant positive linear relationship was found between the amount of phantom-limb pain, as measured on the standardized pain-intensity scale, and the amount of cortical reorganization ($r=0.93$, $P<0.0001$; Fig. 2); phantom-limb pain explained almost 85% (adjusted $r^2=0.83$; $F(1,11)=66.89$, $P<0.0001$) of the variance in cortical reorganization. A similar correlation was obtained for the visual analogue phantom pain scale ($r=0.85$, $P<0.001$) and the adjective phantom pain scale ($r=0.86$, $P<0.001$).

The mean shift in the focus of cortical responsivity to facial stimulation was 0.43 cm (s.d. = 0.40, range 0.01–1.00) for the five pain-free subjects, whereas the mean shift (M) for the eight subjects with phantom-limb pain was almost five times as large ($M=2.05$ cm, s.d. = 1.08, range 0.52–3.86; $F(1,11)=9.94$, $P<0.01$) (Fig. 3). None of the measures for stump pain, non-painful stump sensations or non-painful phantom sensations showed a significant relationship to amount of cortical reorganization. The presence, frequency and intensity of telescoping, or the length of the telescoped phantom, as well as reports of facial remapping (which was present in 4 of the 13 subjects) were unrelated to cortical reorganization. None of the five patients without phantom pain reported the experience of pre-amputation pain. However, half of the eight patients with phantom pain had experienced pain before the amputation (three due to traumatic injury, and one due to osteosarcoma); the other half

FIG. 1 Illustration of the method used to determine the amount of cortical reorganization. The centres of the magnetic responses to stimulation on the face and digits are superimposed onto a magnetic resonance image of an individual subject.

METHODS. In all subjects at least the following four sites were stimulated by using light superficial pressure applied via a pneumatic stimulator: (1) the first and (2) the fifth digits of the (intact) hand; (3) the chin below the left corner of the lip (first five subjects) or the lower lip near the left corner of the mouth (eight subjects) and (4) the chin below the right corner of the lips (first five subjects) or the lower lip near the right corner of the mouth (eight subjects). The recording from the lip was found to yield a much better cortical response than the recording from the chin and was therefore preferred in the latter series of recordings. During these four conditions, the sensor array was positioned over the hemisphere contralateral to the site of stimulation. At each site 1,000 stimuli were delivered at an average stimulation rate of 0.5 Hz (interval between stimulus onsets: 500 ± 50 ms). The sequence of sites at which stimuli were presented was varied according to a fixed irregular order across subjects. After each train of 1,000 stimuli, the subjects indicated the primary and secondary (if any) location of the perceived stimulations, as well as its quality and intensity. Using a BTi neuromagnetometer, magnetic fields were recorded from 37 locations over a circular concave area (14.4 cm diameter) above the parietotemporal cortex contralateral to the site of the stimulation. Recordings were carried out in a magnetically shielded room. Subjects lay in a lateral position with their whole body supported by vacuum cushions. The MEG was sampled at a rate of 520.5 Hz. The evoked magnetic responses from each stimulation site were averaged (from -100 to $+250$ ms) and digitally filtered with a bandpass of 0.01 to 100 Hz. To exclude artefacts, a response was omitted from the average if its range exceeded 2 pT in any of the MEG channels. For each magnetic field distribution, a single equivalent current dipole (ECD) model (best-fitting local sphere) was fitted within the latency range from 35 to 75 ms. From the points with a goodness-of-fit larger than 0.95 and a confidence volume smaller than 300 mm^3 , the region with the maximal field power (measured as root-mean-square across channels) was selected. To illustrate the measure of reorganiza-



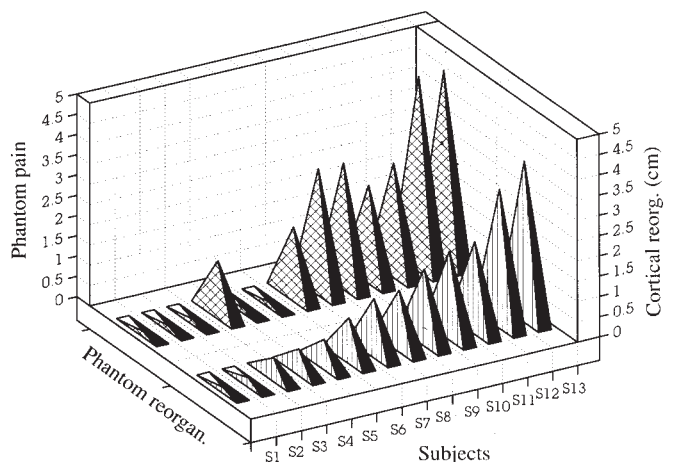
tion used, these ECD locations were mapped onto the cortical surface of area 3b, which was reconstructed from a magnetic resonance image scan as described²⁰. Distances between locations along the curved surface of the somatosensory map can be represented by the distance in the three cortical dimensions (black rods). The arrows connecting the cortical representation of the lip and (1) the midpoint between digits 1 and 5 on the intact side (lower arrow) and (2) the midpoint between the mirror images of the first and fifth digit and the lip on the amputated side (upper arrow), represent the cortical-distance measure used. The mirror images of digits 1 and 5 were obtained by projecting the centres of magnetic activity on the intact side across the midsagittal plane onto the opposite hemisphere (representing the amputation side of the body). To obtain an estimate of the extent of the reorganization that had occurred, a comparison was made of the difference in the two distance measures (that is, the mean coronal shift in the dipole of the amputation-side face area relative to the fingers).

had never experienced pre-amputation pain (Yates-corrected χ^2 , NS). The overall correlation between pre-amputation pain and phantom pain was thus only moderate and not significant. There was a trend towards a significant relationship between the amount of cortical reorganization and pre-amputation pain ($r = 0.51$, $F(1,11) = 3.91$, $P = 0.07$) which was entirely due to the positive though nonsignificant relationship of phantom-limb pain and pre-amputation pain (partial correlation of reorganization and pre-amputation pain with phantom-limb pain removed: $r = -0.132$). Age of patients at time of testing, age at time of amputation, and time elapsed since the amputation were unrelated to both phantom-limb pain and cortical reorganization (all P values NS). A final stepwise regression analysis, with all pain, sensation, demographic and clinical variables entered to predict the amount of cortical reorganization, yielded a single predictor: amount of phantom-limb pain experienced by the subjects.

The results of this study reveal a strong positive relationship between the amount of cortical reorganization occurring after unilateral upper-extremity amputation and the intensity of phantom pain. The present findings suggest the intriguing possibility that the shift of the cortical map following amputation might be a potential neurophysiological basis of phantom-limb pain. The result contradicts current influential theoretical analyses of phantom-limb pain^{2,3}, in which an inverse relationship between painful phantom-limb phenomena and cortical reorganization was postulated, but is consistent with Melzack's³ assumption of a 'neuromatrix' underlying phantom-limb pain. The involvement of cortical reorganization in phantom-limb pain is not inconsistent with the extensive evidence that peripheral mechanisms also contribute to phantom pain^{1,2}. The remodelling of the functional architecture of the cortex in response to nervous-system damage could serve as an adaptive compensatory mechanism by restor-

FIG. 2 Amount of cortical reorganization (in cm) for each subject plotted against intensity of phantom-limb pain, as measured with the Multidimensional Pain Inventory.

METHODS. Phantom-limb and stump pain were assessed by three methods: (1) the pain intensity scale of the West Haven-Yale Multidimensional Pain Inventory^{21,22}, a reliable and valid measure of the amount of pain experienced, administered separately for phantom and stump pain; (2) a phantom-and-stump phenomena interview that included the pain experience scale (Schmerzempfindungsskala²³) consisting of 24 pain adjectives derived from the McGill pain questionnaire²⁴. Each of these 24 descriptors was scored on a 4-point scale according to the extent to which it accurately described the subjects' pain experience. The scale was administered separately for phantom-limb pain, stump pain, and pre-amputation pain; (3) a 10-cm visual analogue scale with the endpoints 'no pain' and 'unbearable pain'. Non-painful phantom phenomena (such as telescoping) and non-painful stump sensations (such as itching, pressure) were measured in the phantom-and-stump phenomena interview. The presence of facial remapping was assessed by probing the surface of the entire body with a cotton-wool bud while the patient indicated the presence of phantom sensations^{8,11}.



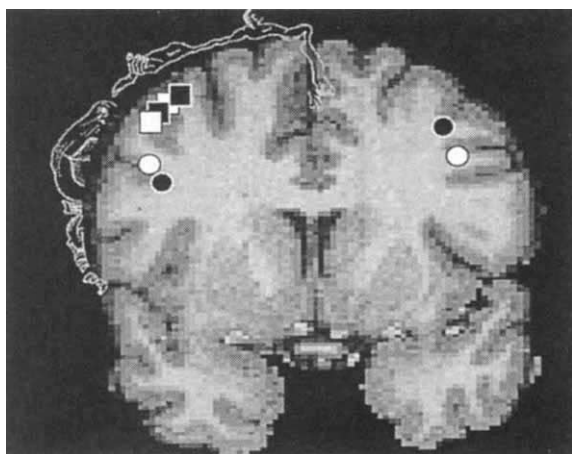


FIG. 3 A representative subject with intense phantom-limb pain (black symbols with white surround) and one subject who experienced no phantom-limb pain (white symbols with black surround) were selected from the patient sample. Squares represent the ECD locations of the digits, circles represent the location of the lip projected onto a coronal section of the brain. Hemisphere contralateral to intact side (left) and amputation side (right). A pronounced hemispheric asymmetry in the location of the lips can be observed in the phantom-pain patient.

ing activity in a zone deprived of its afferent input and could provide a basis for recovery of function. In patients with phantom-limb pain, this mechanism would appear to have become maladaptive, possibly related to a lasting hyperexcitability of nociceptive pathways induced by previous pain¹⁴, imbalance of nociceptive and non-nociceptive inputs after deafferentation¹⁵, or similar mechanisms. The reorganization observed in some subjects with phantom-limb pain (exceeding 3 cm in two subjects) probably encompasses the full extent of the cortical area 3b representing the hand and lower arm area before amputation. It is possible that reorganization may have occurred in additional areas of SI as well as SII, which would not have been assessed by our methods. It has been suggested^{5,16} that the reorganization could be due to synaptic remodelling within the arborization zones of thalamocortical neurons projecting to areas of somatosensory cortex that had retained their normal sources of input and which were adjacent to the affected cortical areas that had lost their afferents (cortical face or head areas). This mechanism, however, can account for only a small fraction of the full topographic extent of the massive reorganization in the present study. To what extent chronically altered afferent inputs produced by sprouting¹⁷, increase in synaptic strength¹⁸ or unmasking of intracortical connections¹⁹ contribute to the reorganization observed here needs to be investigated further. In addition, determination of behavioural methods or pharmacological agents affecting cortical reorganization might eventually be effective in relieving phantom-limb pain. □

Received 14 February; accepted 29 March 1995.

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ACKNOWLEDGEMENTS. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

Essential role of neocortical acetylcholine in spatial memory

J. Winkler^{*†}, S. T. Suhr^{*}, F. H. Gage^{*},
L. J. Thal^{*†} & L. J. Fisher^{*†}

^{*} Department of Neurosciences, University of California, San Diego, La Jolla, California 92093, USA

[†] Department of Neurology, Veterans Affairs Medical Center, San Diego, La Jolla, California 92161, USA

THE cholinergic system plays a crucial role in learning and memory. Lesions of cholinergic nuclei^{1–4}, pharmacological manipulations of cholinergic systems^{5–8}, intracerebral transplantation of fetal tissue^{9–11} and anatomical changes in cholinergic pathways during ageing^{12–14} have all been correlated with altered cognitive behaviour. However, it has not been proved that regional acetylcholine is causally required for learning and memory. Here we describe how we achieved a permanent and selective impairment of learning and memory by damaging the nucleus basalis magnocellularis, a nucleus that provides the major cholinergic innervation of the neocortex^{15,16}, in adult rats. To test the hypothesis that acetylcholine is essential for restoration of cognitive function, we implanted genetically modified cells that produce acetylcholine¹⁷ into denervated neocortical target regions. After grafting, rats with increased neocortical acetylcholine levels showed a significant improvement in a spatial navigation task. Acetylcholine is thus not only necessary for learning and memory, as previously argued, but its presence within the neocortex is also sufficient to ameliorate learning deficits and restore memory following damage to the nucleus basalis.

Adult, male Fischer 344 rats with bilateral ibotenic acid (IBO) lesions of the nucleus basalis magnocellularis (NBM)¹⁸ received transplants of primary fibroblasts genetically modified to express either LacZ (Bgal fibroblasts) or *Drosophila* choline acetyltransferase (dChAT fibroblasts) into the frontal and parietal cortices. dChAT fibroblasts show high levels of ChAT activity and produce and release acetylcholine (ACh), whereas Bgal fibroblasts neither express ChAT nor synthesize ACh (ref. 17). Non-grafted rats with NBM lesions and age-matched unoperated rats were used as additional control groups.

Rats were given a broad range of behavioural tests between one and four weeks after grafting. First, rats were tested in the Morris water maze, a task considered to reflect spatial learning and memory. Over 10 days, all groups learned to find a hidden platform as indicated by decreasing escape latencies (Fig. 1a). Non-grafted or Bgal rats with NBM lesions showed significantly longer mean escape latencies than rats implanted with dChAT fibroblasts ($P < 0.05$) or intact controls ($P < 0.0005$; Fig. 1b). Escape latencies of dChAT rats reached the level of intact controls on the last day of acquisition (Fig. 1a), but were significantly longer than normal across all trials ($P < 0.05$; Fig. 1b).

† To whom correspondence should be addressed at The Salk Institute, Laboratory of Genetics, PO Box 85800, San Diego, California 92186-5800, USA.

Different latencies between groups did not reflect motor disturbances, because swimming speeds were identical for all groups (Table 1).

One day after the last acquisition trial, a probe trial was conducted to assess the spatial acuity of the rats. Both Bgal and non-grafted NBM rats showed random swimming patterns (Fig. 1c) and short swimming distances in the target region around the previous platform location (Fig. 1e). In contrast, dChAT rats displayed focused searching for the platform in the correct location (Fig. 1d), matching the behaviour of the intact control rats (Fig. 1e). Results from the acquisition and probe trials indicate that NBM-lesion-induced deficits in spatial learning and memory are markedly reversed by bilateral cortical grafts of dChAT fibroblasts. None of the other behavioural indices

assessed, including spontaneous activity, passive-avoidance and acoustic startle, was affected either by the lesion or by the cortical grafts (Table 1), suggesting that dChAT fibroblasts do not have a global impact on behaviour. Other experiments in which dChAT fibroblasts were implanted into the intact neo-cortex also have shown no behavioural effect of the grafts (unpublished observations), further suggesting that dChAT cells only affect spatial navigation when grafted in denervated cortical regions.

Four weeks after grafting, all behaviourally tested animals were killed and a random subset of rats from each group was processed for histological, biochemical or molecular analyses of the lesions and grafts. Grafts in the frontal (Fig. 2a, b) and parietal (Fig. 2c, d) cortices were present in all transplanted

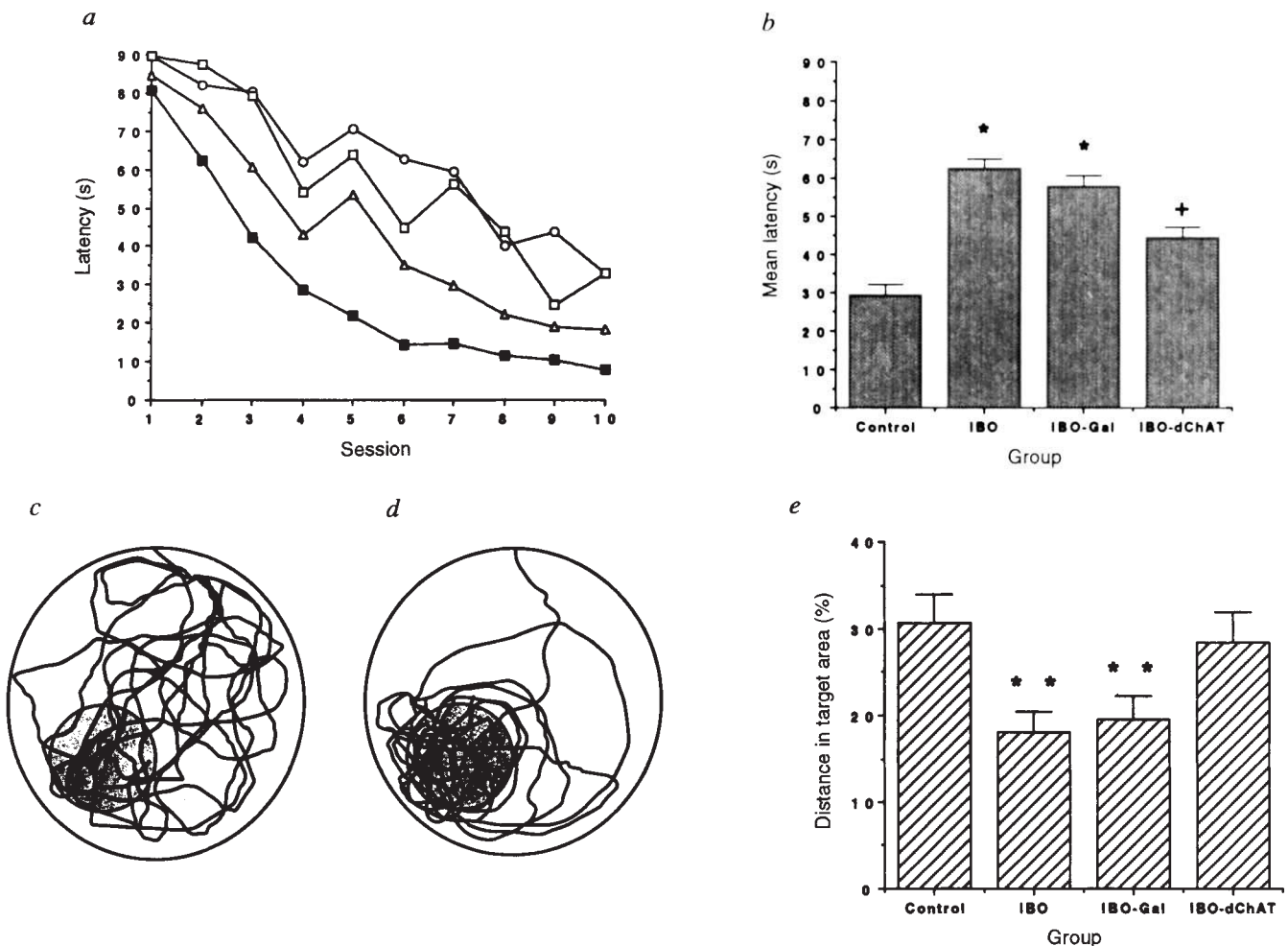


FIG. 1 Spatial navigation in the Morris water maze. **a**, All groups learned the position of the platform, as indicated by the decrease in escape latencies over time. Unlesioned rats (closed squares; $n=10$) displayed the shortest latencies, followed by rats implanted with the dChAT fibroblasts (open circles; $n=10$). The IBO only (open circles; $n=10$) and IBO-Bgal (open squares; $n=10$) groups consistently showed the longest escape latencies. Each point represents the average of two trials. **b**, Mean escape latencies during the 10-day acquisition period. IBO-dChAT rats showed mean latencies that approached, but remained significantly longer than, the mean latencies of intact rats (+, $P<0.05$ between groups). The dChAT group found the platform significantly faster than both IBO-Bgal and IBO animals (*, $P<0.02$). **c**, **d**, Representative swim paths of rats in the Bgal (**c**) or dChAT groups (**d**) in the spatial probe trial. Starting point is north (top). The circle (29-cm radius) marks the target region around the previous platform location and represents ~12% of the pool area. The swim path of the dChAT animal is focused near the platform whereas the Bgal rat shows a random search pattern. **e**, Average swim distance in platform target area. dChAT grafted animals showed a spatial acuity that matched the intact, control rats and

was significantly better than Bgal grafted and non-grafted NBM-lesioned animals (**, $P<0.05$ compared with intact and dChAT rats).

METHODS. The maze consists of a circular fibreglass pool (diameter: 1.52 m) filled with water at a temperature of 19–20 °C. Rats received two trials (90 s) per day for two consecutive 5-day periods separated by 2 days⁸. One day after the last acquisition trial, rats were tested in a spatial probe trial in which rats swam freely in the pool for one 90-s period in the absence of the platform. Differences between groups were evaluated by ANOVA. Repeated-measures ANOVA was used for the analysis of acquisition (group $\times$ days). There were significant between-group differences for latency ($F(3,36)=13.99$, $P<0.0001$) and distance ($F(3,36)=13.0$, $P<0.0005$). All groups showed a significant improvement in latency over time ($F(9,324)=67.29$, $P<0.0001$). Mean latency was analysed by factorial one-way ANOVA followed by Fisher's least squares difference *post hoc* test. Distances in target area (%) were analysed by factorial one-way ANOVA, followed by Fisher's least squares difference *post hoc* test. There was a significant group difference ($F(3,36)=4.40$, $P<0.01$). Data are expressed as means $\pm$ s.e.m.

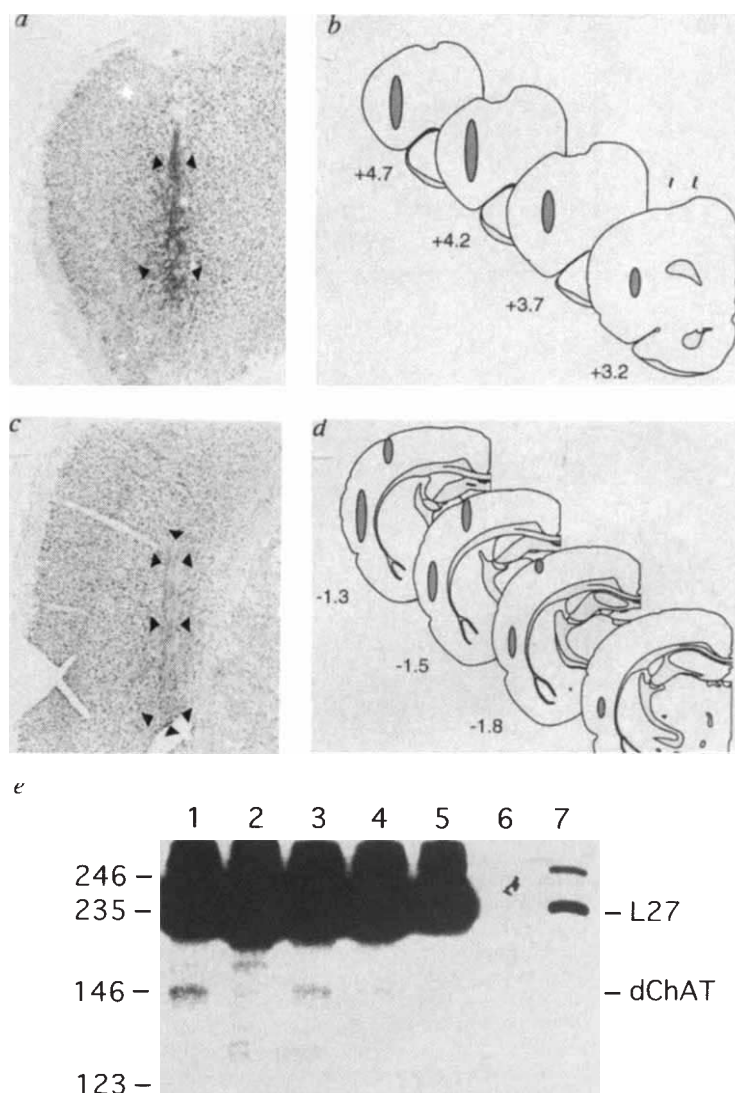
TABLE 1 Additional behavioural assessments of grafted and non-grafted rats

Groups	Mass (g)	Swim speed (m s ⁻¹)	Open field activity no. of crossings	Passive avoidance 72-h retention (s)	Startle (motor units)
Control	279.5 ± 2.9	0.38 ± 0.01	60.2 ± 5.6	281.5 ± 16.2	1,097.1 ± 176.0
IBO lesion	262.3 ± 4.9*	0.38 ± 0.01	59.3 ± 8.6	294.8 ± 5.2	939.2 ± 112.0
IBO/Bgal	261.6 ± 3.3*	0.39 ± 0.01	68.7 ± 10.2	276.2 ± 17.7	1,133.0 ± 156.2
IBO/dChAT	265.6 ± 4.4*	0.37 ± 0.01	60.4 ± 10.6	286.9 ± 10.5	1,446.5 ± 114.8

Swim speed was determined during the spatial probe trial (m s⁻¹). After water-maze testing, rats were sequentially tested for spontaneous motor activity in an open field (19 days after grafting), passive avoidance (20–26 days after grafting) and startle (27 days after grafting). Motor activity was measured in an 81-cm² box marked with a grid of 20-cm squares; the number of grid lines crossed was counted for a 5-min period. Passive avoidance was conducted as described elsewhere⁹. Startle reflexes were assessed by measuring the maximal motor response of rats to 115 dB stimuli (50 ms duration) during eight blocks of six consecutive trials/block spaced 15 s apart (total of 48 trials). Activity was measured by a piezoelectric accelerometer and recorded in arbitrary motor units (San Diego Instruments). Average rat mass was obtained at the time of killing (28 days after grafting). All rats with NBM lesions were healthy but weighed significantly less than unlesioned control rats (analysis of variance (ANOVA): $F(3,36) = 4.48$, $P < 0.01$). *, $P < 0.05$ compared with control (Fisher's least squares difference *post hoc* test). The masses of grafted and non-grafted rats with NBM lesions did not differ from each other ($P > 0.05$). Data expressed are means ± s.e.m.

FIG. 2 Representative photomicrographs and schematic reconstruction of fibroblast grafts within the neocortex. *a, c*, Coronal section stained with thionin showing a graft implanted into the frontal (*a*) and parietal (*c*) neocortex. *b, d*, Representative reconstructions of graft localization within the frontal (*b*) and parietal (*d*) neocortex. The numbers by the sections indicate the approximate anterior–posterior (AP) coordinates of the sections with respect to bregma²⁹. The grafts are indicated by light shading. *e*, RT–PCR analysis of microdissected grafted and non-grafted cortical regions. Samples collected from the frontal (lanes 1 and 3) and parietal (lanes 2 and 4) cortices of two behaviourally tested rats implanted with dChAT fibroblasts show PCR product for dChAT mRNA (146 bp). No band was detected either in non-grafted cortical regions (lane 5) or in the absence of RT (lanes 6 and 7), confirming both the localization and continued transcription of the transgene in the dChAT fibroblasts four weeks after grafting. The ribosomal protein L27A (L27, 235 bp) was used as an internal control for the total amount of mRNA and efficiency of reverse transcription in each reaction.

METHODS. Adult male Fischer 344 rats ($n = 30$) received bilateral lesions of the NBM by infusion of IBO as previously described¹⁸. One week after the second NBM lesion, 0.5 µl of dChAT or Bgal fibroblasts (100,000 per µl) were slowly infused into six bilateral regions of the frontal (AP: +12.2, ML: ±2.0, DV: +8.0; AP: +12.2, ML: ±3.5, DV: +6.0, +7.5 mm from the interaural plane) and parietal (AP: +6.7, ML: ±4.2, DV: +7.2; AP: +6.7, ML: ±6.0, DV: +4.4, +6.2 mm from the interaural plane)²⁹ neocortex. The dChAT fibroblasts used in the present study showed a ChAT activity of 565.4 ± 6.7 nmol ACh h⁻¹ per mg protein and ACh levels of 118.1 nM and 18.1 nM in cell homogenates and extracellular media, respectively. Four weeks after grafting, 4 or 5 rats from each group were perfused with 4% paraformaldehyde, and 40-µm sections through the brain were processed for thionin and Bgal or dChAT protein as previously described^{11,17}. For PCR analyses, animals were killed by decapitation. Brains were rapidly removed under sterile conditions, and grafted and non-grafted cortical areas were dissected, under microscopic guidance, on ice and processed for detection of dChAT mRNA by using standard guanidinium–thiocyanate–CsCl gradient purification methods. Total RNA (10 µg) was reverse-transcribed in 1 × PCR buffer (10 mM Tris–Cl, pH 8.3, 50 mM KCl), 2.5 mM MgCl₂, 1 mM dNTP, 100 pM random hexamers, 20 units RNasin, and 12.5 units AMV reverse transcriptase in a 20 µl volume for 75 min at 42 °C followed by 10 min at 95 °C. PCR amplification was performed by adding premixed 1 × PCR buffer in water, additional MgCl₂ (1.75 mM final concentration), 2×10^{-3} mCi [³²P]dCTP, 2.5 units Taq polymerase, and 0.5 µg of each primer. Of this mix, 80 µl was added to each RT reaction for a total of 100 µl. Samples were overlaid with mineral oil and amplified in a PEC thermocycler 480 as follows: 2 min at 95 °C, 2 min at 60 °C, and 2 min at 72 °C, for 30 cycles. A 40-µl sample of reaction product was analysed by 6.5% PAGE



Primer sequences were: dChAT-forward (F) primer, 5'-CTGGA-GAAGCGTGAGGCGAG; dChAT-reverse (R) primer, 5'-GCACGTCGTGGAC-GGTCTTG; ribosomal protein L27A F-primer, 5'-ATCGGT-AAGCACCAGCAAGCA; RPL27A R-primer, 5'-GGGAGCAACTCCATTCTGT. DChAT primers were designed and tested for non-cross-hybridization with endogenous rat ChAT (results not shown). RPL27A PCR product in the absence of RT (lane 7) presumably reflects the presence of small amounts of genomic DNA.

TABLE 2 Biochemical assessments of grafted and non-grafted tissues

(a)			
Group	Frontal cortex	Parietal cortex	Olfactory bulb
Control (n = 5)	53.2 ± 1.8	43.9 ± 1.4	30.7 ± 0.6
IBO (n = 6)	38.6 ± 1.4**	39.9 ± 0.7**	29.1 ± 0.5
IBO/Bgal (n = 5)	40.2 ± 1.6**	39.4 ± 1.3**	28.7 ± 1.1
IBO/dChAT (n = 6)	41.8 ± 1.3**	40.2 ± 0.9**	28.3 ± 0.4
(b)			
Group	ACh	Choline	
Control (n = 5)	276.8 ± 93.5	780.9 ± 316.1	
IBO (n = 5)	181.5 ± 148.2	339.8 ± 200.3	
IBO/Bgal (n = 4)	259.1 ± 91.9	1,064.2 ± 238.4	
IBO/dChAT (n = 3)	1,310.5 ± 316.6**	1,802.8 ± 258.4	

a, ChAT activity in frontal and parietal cortices of grafted and non-grafted rats. b, Levels of acetylcholine (ACh) and choline in cortical grafts and comparable cortical areas of non-grafted lesioned and unlesioned animals. A representative subgroup of behaviourally tested rats from each group received intraperitoneal injections of physostigmine salicylate (0.32 mg kg⁻¹) 30 min before decapitation, to inhibit the activity of acetylcholinesterases⁷. The brains were rapidly removed, and non-grafted cortical areas, grafted cortical regions and the olfactory bulbs were carefully dissected, under microscopic guidance, on ice. Grafted and non-grafted cortical regions were processed for ChAT activity or ACh and choline as previously described^{7,17,18}. Olfactory tissues were processed for ChAT activity only. ChAT activity was analysed by two-way ANOVA (group × region) followed by Tukey *post hoc* test; results are expressed as means ± s.e.m. in nmols ACh formed h⁻¹ per mg protein (frontal and parietal values pooled). The effect of the NBM lesion on ChAT activity was significant ($F(3,124) = 45.41$, $P < 0.0005$). **, $P < 0.0005$ compared with control. ChAT activity in the olfactory bulb was not affected by the lesion ($P > 0.05$). Because microdissected dChAT grafts did not show greater ChAT activity than in control groups, experiments were conducted *in vitro* in which several concentrations of dChAT fibroblasts were added to microdissected regions of the neocortex. Results indicated that the ChAT activity of <300,000 dChAT fibroblasts could not be detected above the background ChAT activity in the cortical homogenates. Concentrations of ACh and choline were analysed separately by one-way factorial ANOVA, followed by the Tukey *post hoc* test. ACh concentrations between groups were significantly different ($F(3,18) = 10.36$, $P < 0.0005$). Values shown are pooled samples from the frontal and parietal grafts and expressed as means ± s.e.m. in fmol ACh or choline per 20 µl sample. **, $P < 0.0005$ compared with unlesioned controls, non-grafted IBO rats and Bgal animals. Choline levels between groups were not significant ($F(3,18) = 2.89$, $P = 0.064$).

animals. Bgal grafts showed Bgal immunoreactivity (not shown), whereas specific staining for dChAT protein in dChAT grafts was obscured by high labelling in the surrounding host tissue¹⁷. ChAT activity in dissected cortices from non-grafted IBO rats revealed cholinergic depletions ranging from 9% to 27% in the parietal and frontal cortices, respectively ($P < 0.0005$; Table 2a). Non-grafted cortical regions dissected from Bgal and dChAT rats showed similar decreases in ChAT levels, indicating that the different water-maze performances of the groups did not reflect variations in cholinergic damage. Good localization of the toxin to the NBM was suggested by the normal amounts of ChAT activity within the olfactory bulb, an area that receives only minor cholinergic input from the NBM^{15,16} (Table 2a). ChAT activity in microdissected dChAT grafts did not differ from that in Bgal grafts or non-grafted cortical areas ($P > 0.05$). Subsequent experiments *in vitro* indicated that the activity of 150,000 dChAT fibroblasts implanted in the cortex was below the detection limit of the ChAT assay (Table 2a).

Expression of the dChAT transgene *in vivo*, assessed by using reverse transcriptase (RT)–polymerase chain reaction (PCR), revealed dChAT mRNA only in microdissected grafts from the dChAT group (Fig. 2e). The amount of bioactive ChAT protein within the grafted fibroblasts was then determined by measuring ACh levels within grafted and non-grafted cortices. Before the rats were killed they were injected with physostigmine to block endogenous acetylcholinesterase activity and enhance the detection of ACh. DChAT grafts contained 4- to 6-fold more ACh ($P < 0.0005$; Table 2b) than Bgal grafts or corresponding cortical areas in non-grafted NBM and control rats. Because of the use of physostigmine, these results do not show the endo-

genous amount of ACh but rather that there is a relative increase of ACh in the dChAT animals compared with the control groups. Because the only difference between the Bgal and dChAT fibroblasts is the production and release of ACh by the dChAT fibroblasts, the improved performance of dChAT rats in spatial navigation can be directly linked to the increased intracortical ACh in these animals.

Our results are consistent with previous observations that transplants of cholinergic-rich fetal tissue in the neocortex reverse deficits in learning and memory following excitotoxic lesions of the NBM^{9,19}. We have further shown that the replacement of ACh alone within the denervated neocortex is sufficient for improving cognitive function. This improvement is achieved regardless of which neurotransmitter or neuroanatomical systems^{20,21} contribute to the lesion-induced deficit. Second, the data suggest that successful performance of spatial navigation may not require regulated cholinergic activity within the neocortex. Fibroblasts seem to have the capacity for quantal secretion of ACh²², but the released ACh presumably acts in a tonic manner on postsynaptic receptors, as postulated for dopamine released from grafted non-neural cells^{23,24}. However, residual presynaptic cholinergic fibres near the dChAT grafts may capture the additional ACh and/or choline and release it in an appropriate spatio-temporal pattern. Last, the data support a crucial, but not exclusive, role of the neocortical circuitry in spatial memory. Because excitotoxic lesions of the NBM disrupt attention²⁵, it remains to be clarified whether the dChAT graft-induced improvements in spatial memory reflect an effect of cortical ACh on attention or on other components of learning and memory. So far, insights into learning and memory^{1,2,21,26–28} have been gained primarily in the form of correlations rather than causal relationships between molecules and complex systems. In the present study, damage of the NBM produced a selective cognitive impairment that was corrected by site-specific enhancement of ACh in the neocortex. These results thus confirm the hypothesis that the presence of acetylcholine in the neocortex is essential for recovery of spatial memory following basal forebrain damage. □

Received 28 December 1994; accepted 12 April 1995.

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ACKNOWLEDGEMENTS. We acknowledge the technical assistance of J. Anderson, A. Chen, H. Grajeda, B. Miller and M. Wardlaw. J.W. is a fellow of the McDonnell–Pew Center for Cognitive Neuroscience at San Diego (Salk Institute). This work was supported by grants from the Veterans Administration, NIA, NINDS, Margaret and Herbert Hoover Foundation, Metropolitan Life and AlliedSignal.

Essential functions of synapsins I and II in synaptic vesicle regulation

Thomas W. Rosahl^{*†}, Diane Spillane[‡],
Markus Missler^{*†§}, Joachim Herz^{*},
David K. Selig[‡], Joachim R. Wolff[§],
Robert E. Hammer^{†||}, Robert C. Malenka[‡]
& Thomas C. Südhof^{*†||}

Department of ^{*} Molecular Genetics and ^{||} Biochemistry, and [†] Howard Hughes Medical Institute, The University of Texas Southwestern Medical School, Dallas, Texas 75235, USA
[‡] Departments of Psychiatry and Physiology, Center for Neurobiology and Psychiatry, University of California at San Francisco, San Francisco, California 94143, USA
[§] Zentrum Anatomie, Universität Göttingen, 37075 Göttingen, Germany

SYNAPTIC vesicles are coated by synapsins, phosphoproteins that account for 9% of the vesicle protein¹⁻³. To analyse the functions of these proteins, we have studied knockout mice lacking either synapsin I, synapsin II, or both. Mice lacking synapsins are viable and fertile with no gross anatomical abnormalities, but experience seizures with a frequency proportional to the number of mutant alleles. Synapsin-II and double knockouts, but not synapsin-I knockouts, exhibit decreased post-tetanic potentiation and severe synaptic depression upon repetitive stimulation. Intrinsic synaptic-vesicle membrane proteins, but not peripheral membrane proteins or other synaptic proteins, are slightly decreased in individual knockouts and more severely reduced in double knockouts, as is the number of synaptic vesicles. Thus synapsins are not required for neurite outgrowth, synaptogenesis or the basic mechanics of synaptic vesicle traffic, but are essential for accelerating this traffic during repetitive stimulation. The phenotype of the synapsin knockouts could be explained either by deficient recruitment of synaptic vesicles to the active zone, or by impaired maturation of vesicles at the active zone, both of which could lead to a secondary destabilization of synaptic vesicles.

Independent lines of mice with mutations in the synapsin II gene were generated by homologous recombination and bred to homozygosity. The two strains were crossed with two independent strains of synapsin-I knockout mice⁴, resulting in the generation of two independent knockout lines each for synapsin I, synapsin II, or both synapsins. Genetically matching wild-type controls were bred in the same crosses. Immunoblotting of total brain protein from different strains of mutant mice demonstrated

stained sagittal sections of whole brains. Regional landmarks identified in the left panel are: ob, olfactory bulb; Ocl, occipital cortex; h, hippocampus; cb, cerebellum; dcn, deep cerebellar nuclei. In *d*, brain regions used for qualitative and quantitative electron microscopy are circled and boxed, respectively. *e*, Representative electron micrographs of cerebral cortex from wild-type (WT) and double-knockout mice (DKO). Arrows point to typical chemical synapses having a normal structure overall but a relative depletion of synaptic vesicles. Arrowheads delineate synaptic vesicle clusters. Scale bar, 10 μ m. *f*, Distribution of synaptic vesicles in nerve terminals from wild-type and double-knockout mice. The distances of synaptic vesicles from the active zone were measured in sections of 20 representative nerve terminals.

METHODS. *a*, Generation of knockouts. A genomic clone encoding exons 8–11 of the murine synapsin II gene was isolated to construct a knockout vector in which exons 9 and 10 are replaced by a neomycin-resistance marker and which contains two HSV-TK genes for negative selection (data not shown)^{4,22,23}. The knockout vector was transfected into ES cells generated from SV129 mice. Clones obtained after positive and negative selection with G418 and FIAU were analysed by PCR (oligonucleotide sequences: GAACCAACTGGTTCCTATCACTAGCTA and GGATGCGGTGGGCTCTATGGCTTCTGA). Homologous recombination was observed at a frequency of 1:5 and confirmed by Southern blotting (data not shown). One of the three ES cell clones carried a duplication of the homologously recombined sequence but still completely abrogated expression. ES cell clones carrying the mutation were transferred into blastocysts, chimaeric mice were bred, and two mouse lines with genetically matching wild-type controls were established. Different synapsin-II-knockout lines were crossed with previously obtained synapsin-I-knockout lines⁴ to obtain two independent double-knockout lines from four independent ES cell clones. All lines were analysed by PCR, Southern blotting and immunoblotting^{4,13,21}. Synapsin I and II expression were analysed with monoclonal antibodies against these proteins (gift from R. Jahn) and with polyclonal antibodies against the N terminus of all four synapsins, or against synapsin I or II-specific epitopes raised against full-length synapsin I or a GST-synapsin II fusion protein containing the C-domain of synapsin II³. All studies reported here, apart from those measuring seizure frequencies, were performed on animals before seizures developed. *b*, Measuring seizure incidence. 195 mice of all 9 possible combinations of wild-type and mutant alleles were tested blindly at 10 weeks of age in 20 observation days. Mice were moved up and down 30 times in ~30 s, suspended by their tails over a distance of 30 cm, and then placed in a new cage⁵. Seizures were scored before, during or immediately after the stimulation. After completion of the experiments, genotypes were determined and they were grouped as shown. No significant difference was detected between mouse lines derived from different ES cell clones. *c*, Morphological analysis. Anaesthetized animals were perfused transcardially at ~2 ml min⁻¹ with 15 mM PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (light microscopy), or with 0.1 M phosphate buffer, pH 7.4, followed by 3% paraformaldehyde, 3% glutaraldehyde in 0.1 M phosphate buffer (electron microscopy). Serial sagittal sections were cut on a freezing microtome (30 μ m) and analysed by standard histological procedures, with sections from wild-type and mutant animals processed simultaneously under identical conditions. For immunocytochemistry, brains were postfixed in the respective fixative for 2 h and cryoprotected in 25% sucrose overnight. Sections were treated with 0.5% Triton X-100 for 5–20 min, blocked overnight in 2% normal goat serum in PBS, and reacted with antibodies against most of the proteins listed in Table 1 and against the InsP₃ receptor, neuron-specific enolase, phosphorylated and non-phosphorylated neurofilaments H, tyrosine hydroxylase, parvalbumin, glial fibrillary protein, S100 protein and tenascin, and with lectins followed by antibodies against these lectins (*Vicia villosa* agglutinin, *Griffonia simplicifolia*, soybean lectin and *Ulex europaeus*). Bound antibodies were visualized by the peroxidase method with diaminobenzidine and heavy-metal enhancement²⁴. For electron microscopy, dissected brains were postfixed overnight in 3% paraformaldehyde, 3% glutaraldehyde in 0.1 M phosphate buffer, treated with 1% OsO₄ for 1 h at 4 °C, and embedded in epon 812. Matching areas from the olfactory bulb, cerebral cortex (Ocl), hippocampus (CA3 and CA1), cerebellar cortex (vermis), and deep cerebellar nuclei were identified in semithin sections (1.5 μ m) stained with methylene blue azur II. Ultrathin sections (60 nm) were stained with uranyl acetate (20 min) and lead citrate (7 min), and examined with a Zeiss EM10 electron microscope. To measure the spatial distribution of synaptic vesicles, distances between the centre of vesicles and the active zone were measured in randomly chosen terminals of the CA1 region of the hippocampus, with a class width of 50 nm and expressed in a frequency diagram^{11,12}.

FIG. 1 *a*, Immunoblotting analysis of wild-type mice and synapsin-I, synapsin-II and double-knockout mice. Blots were reacted with the indicated antibodies; GDI (GDP dissociation inhibitor) and Rab3A serve as positive controls. Two independent mouse lines homozygous for mutations in either the synapsin I gene (Syn I KO) or the synapsin II gene (Syn II KO) were generated by homologous recombination in embryonic stem cells; double knockouts (DKO) were then obtained by interbreeding. Both independent lines for each genotype were analysed by blotting as indicated; for quantification of protein levels, see Table 2. *b*, Dosage-dependent incidence of epileptic seizures in mice carrying mutations in synapsin genes. The genotype is indicated on the x-axis for female (top line) and male mice (bottom line). X denotes the synapsin I gene, and N the synapsin II gene. Capital letters indicate wild type and lower-case letters mutant alleles; dashes are positions in which there is no equivalent genotype for male mice because the synapsin I gene is on the X chromosome. The y-axis reports the frequency of seizure induced per vestibular stimulation. *c* and *d*, Morphological analysis of the brain architecture of wild-type and double-knockout mice. Panels depict Nissl-

^{*} To whom correspondence should be addressed.

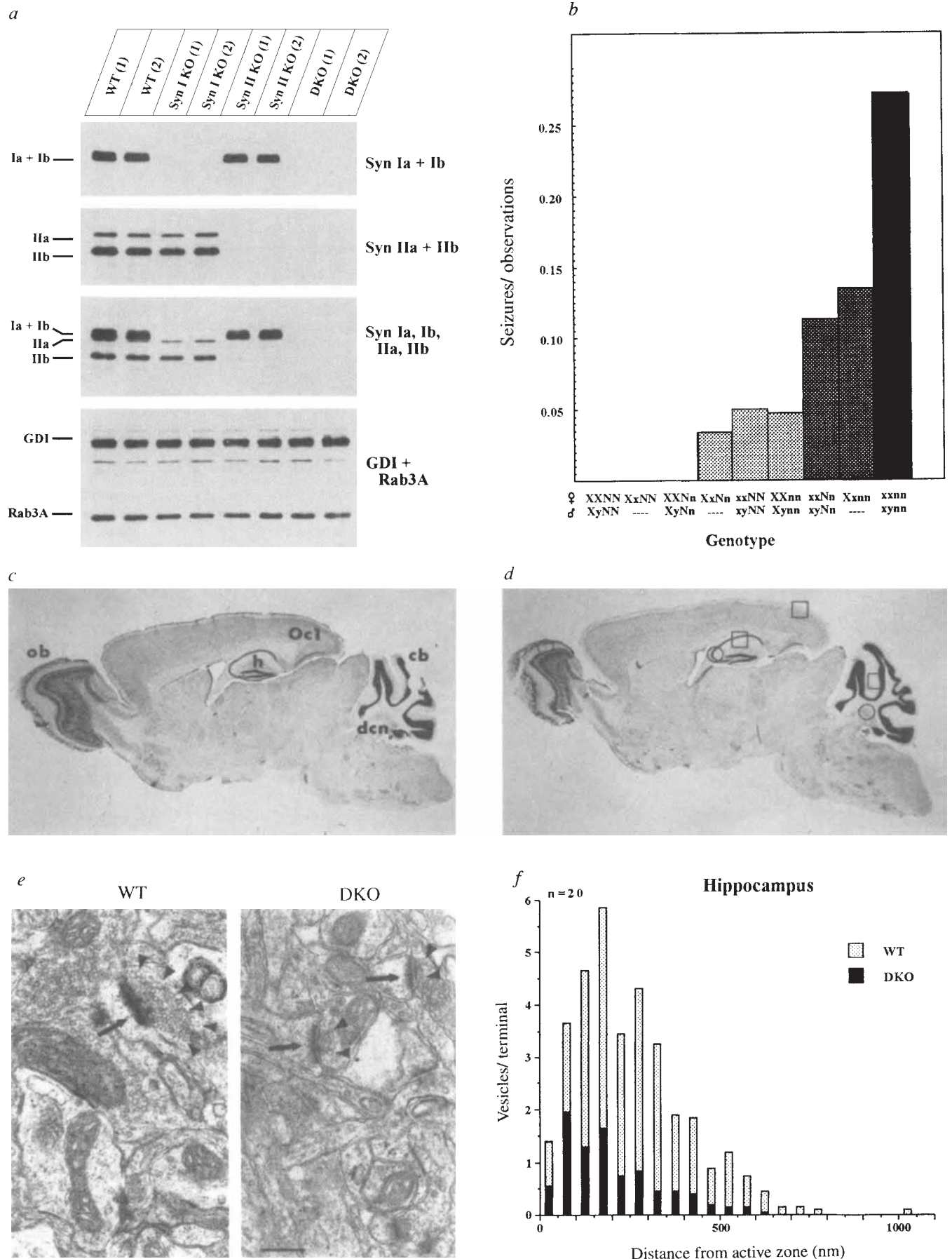


TABLE 1 Ultrastructural analysis of central synapses

	Hippocampus		Visual cortex	
	Wild type	Double knockout	Wild type	Double knockout
Synapse density (type 1) (N 1,000 μm^{-2})	368 $\pm$ 15	344 $\pm$ 26	265 $\pm$ 20	275 $\pm$ 17
Synapse density (type 2) (N 1,000 μm^{-2})	36 $\pm$ 7	43 $\pm$ 8	52 $\pm$ 18	43 $\pm$ 14
Synaptic vesicle density (N μm^{-2})	168 $\pm$ 51	73 $\pm$ 30	173 $\pm$ 61	88 $\pm$ 32
Presynaptic area (10 $^{-2}$ μm^2)	13 $\pm$ 11	11 $\pm$ 06	18 $\pm$ 11	13 $\pm$ 08
Active-zone length (10 $^{-2}$ $\times$ μm)	21 $\pm$ 07	18 $\pm$ 07	23 $\pm$ 09	19 $\pm$ 08

Analyses were done on a series of electron micrographs (Fig. 1 legend) taken at defined distances in the visual cortex (Ocl, lamina 1) and the stratum radiatum of the hippocampal CA1 region (final magnification, $\times 28,000$)¹⁸. Data shown are means $\pm$ s.d. The densities of type 1 and type 2 synapses were determined on photographs representing an area of $\sim 2,400 \mu\text{m}^2$ in each brain region. For quantitation of ultrastructural parameters of synaptic terminals, 50 type 1 synapses with visible synaptic clefts and clear boundaries were randomly selected from hippocampus and visual cortex. Active-zone length (measured as profile length), presynaptic terminal area (measured by a point-counting method¹⁹) and area density of synaptic vesicles were determined. Only values for synaptic vesicle densities were significantly different between double-knockout and wild-type brains, as assessed by Welch's alternative t-test at a significance level of $P < 0.001$ (ref. 20), and are shown in bold typeface.

that each lacks the respective protein whose gene was mutated (Fig. 1a). All single- and double-knockout mice are viable and fertile and have an apparently normal life expectancy (analysed up to 18 months of age). Thus synapsins, in spite of constituting an abundant class of synaptic vesicle phosphoproteins, are not essential for life or overall brain function.

The mutant mice experience no major morbidity apart from seizures that are precipitated by sensory stimuli. The seizures develop only after two months of age and consist of typical *grand mal* attacks with arched backs, inability to stay upright, and post-seizure grooming. To determine the relationship between seizure incidence and genotype, the frequency of seizures induced by vestibular stimulation⁵ was counted by a blinded observer (Fig. 1b). No seizures were observed in wild-type mice or mice heterozygous for the synapsin I or II gene mutation. Mice carrying two or more mutant alleles exhibited an incidence of seizures proportional to the number of mutant alleles, with double knockouts seizing during almost every third trial. Preliminary experiments suggest that the seizure incidence depends on the genetic background, with other backgrounds causing a greater incidence than the SV129/C57 black-6 background used here.

Brains from mice lacking synapsins had a normal size, form, and structure (Figs. 1c and d, and data not shown). Brain sections stained with more than 20 antibodies and lectins were indistinguishable between wild-type and mutant animals by light microscopy (data not shown). In cultured hippocampal neurons, neurite outgrowth appeared to be identical between mutant and wild-type neurons (data not shown). Thus, synapsins are not required for neurite outgrowth, synaptogenesis or the development of a normal synaptic brain architecture.

Selected brain regions of wild-type and double-knockout mice (visual cortex, hippocampus, cerebellum and olfactory bulb) were studied by electron microscopy (Fig. 1e). Overall, the structures and distributions of synapses were similar between wild-type and double-knockout brains except for a relative lack of synaptic vesicles in mutant synapses. To assess this quantitatively, five synaptic parameters were measured in three brain areas in wild-type and double-knockout mice (Table 1 and data not shown). The region-specific densities of type 1 and type 2 synapses were almost identical between wild-type and double-mutant synapses, as were the sizes of the presynaptic terminals and of their active zones. In all brain areas investigated, however, the numbers of synaptic vesicles were dramatically reduced to

$\sim 50\%$ in mutant synapses compared to wild-type synapses (Table 1).

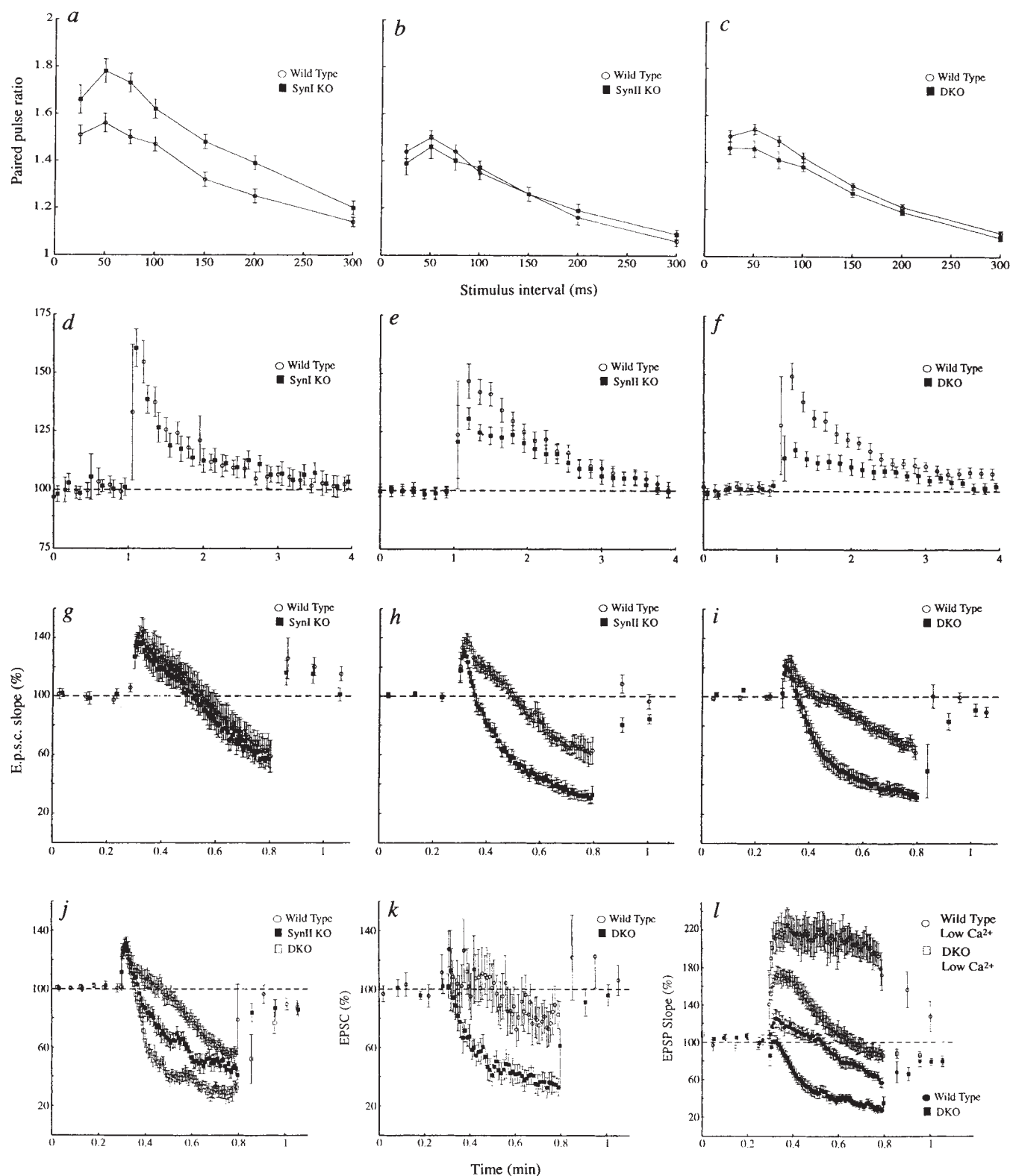
One model for synapsin function proposes that the synapsins attach synaptic vesicles to the cytoskeleton^{6,10}, suggesting that knockout of synapsins should lead to a redistribution of the vesicles. We therefore measured the distances between synaptic vesicles and active zones, a method well suited for determining the three-dimensional distribution of vesicles^{11,12}. No major redistribution of vesicles or 'unclustering' was seen, in particular there was no dispersion of vesicles away from the active zone (Fig. 1f). Thus, knockout of synapsins leads to a depletion of

FIG. 2 a–c, Analysis of paired-pulse facilitation (PPF) in synapsin knockouts. Synapses from wild-type mice (open circles) are compared to synapses from synapsin I (a, synI KO), synapsin II (b, synII KO), and double-knockout mice (c, DKO). d–f, Analysis of post-tetanic potentiation (PTP). PTP was induced by a 1-s 100-Hz stimulus at 1 min. Synapsin-I-deficient synapses exhibit normal PTP (d) but in synapsin-II-deficient synapses (e) and in double-knockout synapses (f), PTP is inhibited. g–i, Synaptic response to repetitive stimulation. At 0.3 min, synapses were stimulated at 10 Hz for 0.5 min. No differences between wild-type and synapsin-I-deficient synapses are observed (g), but synapsin-II-deficient synapses (h) and synapses lacking both synapsins (i) show a much faster and larger depression during the stimulus train than normal synapses. Note that in double knockouts, synaptic depression reaches 50% within 6 s, whereas wild-type synapses show no depression at this point. j, Simultaneous analysis of synaptic depression elicited by repetitive stimulation in wild-type mice, synapsin-II knockouts, and double knockouts. Slices from mice of the three genotypes were interleaved daily to control for interexperimental variability in establishing a rank order of depression. k, Whole-cell voltage-clamp recordings from wild-type and double-knockout cells. Synapses were stimulated at 10 Hz for 0.5 min. l, Lowering extracellular Ca^{2+} reveals that synaptic depression during repetitive stimulation occurs early. Decreasing the probability of release prevents synaptic depression in wild-type synapses, but synapses lacking synapsins still do not maintain transmitter release at control levels throughout the stimulus train. METHODS. Preparation of hippocampal slices, recording procedures at 27–29 °C, data acquisition and analysis were performed as described⁴ without information about the genotype of the mouse under study. To block NMDA-receptor-dependent plasticity, 50 μM D-AP5 was added to the superfusion medium. Male and female age-matched mice of identical genetic background were studied at 4–7 weeks of age. For the PTP experiments, e.p.s.ps were sampled every 3 s. Each point is the average of 3 consecutive responses in d–f, and of 4 consecutive responses in g–i. For each panel except k, data shown were collected from the S number of slices obtained from n number of animals, denoted as S/n: in a, 10/3 for wild-type and 12/4 for mutant synapses; b, 15/4 for both wild-type and mutant synapses; c, 30/9 for both wild-type and mutant synapses; d, 7/2 and 6/2 for wild-type and mutant synapses, respectively; e, 15/4 and 13/4 for wild-type and mutant synapses, respectively; f, 12/4 for both wild-type and mutant synapses; g, 10/3 and 10/4 for wild-type and mutant synapses; h, 12/4 for both wild-type and mutant synapses; i, 17/5 and 18/6 for wild-type and mutant synapses, respectively; j, 12/3 for all three genotypes; k, 6/2 for both genotypes. Whole-cell voltage-clamp recordings (k, 6 cells from 3 wild-type mice and 7 cells from 3 double knockouts) were made using an Axoclamp 2A (Axon Instruments). Only 1 cell per slice was recorded. The pipette solution contained 117 mM Cs-gluconate, 17 mM CsCl, 3.2 mM Mg-ATP, 0.25 mM Na-GTP, 8 mM NaCl, 2 mM MgCl_2 , 0.2 mM EGTA, and 10 mM HEPES-NaOH, pH 7.4. Cells were held at -70 mV with continuous monitoring of input and access resistance. Cells were excluded from data analysis if either parameter changed by more than 10%. Spontaneous and miniature e.p.s.cs are defined by the absence or presence of tetrodotoxin (1 μM). Sucrose-evoked e.p.s.cs were generated by bath application of 0.1 M sucrose for 5 min. To allow for adequate equilibration of the sucrose, data were only analysed for the final 3 min of application. Events (s.e.p.s.cs or m.e.p.s.cs) were selected using software supplied by J. Steinbach. Automated criteria for inclusion as an event included a peak amplitude greater than 3 pA and an initial slope greater than 1.5 pA ms⁻¹. Each computer-selected event was visually inspected and either accepted or rejected. Long-term potentiation, defined as a $>20\%$ increase in e.p.s.p. slope measured 40–50 min after the initiating stimulus, was elicited by a 100-Hz, 1-s tetanus given twice with an interval of 10 s.

synaptic vesicles in nerve terminals without major changes in their distribution or in the general synaptic structure.

Nineteen synaptic proteins were measured in knockout and wild-type mice to test whether the decrease in vesicle numbers in the knockouts is accompanied by a similar reduction in vesicle proteins, and whether such changes can also be observed in single knockouts. Vesicle proteins were slightly decreased in single knockouts, and decreased much more in double knockouts (Table 2). No change was detected in synaptic proteins from the

cytosol or plasma membrane. Because of the small size of the decrease in single knockouts, it only reached statistical significance after analysis of large sample numbers and was not significant in the previous synapsin I knockout study⁴. Surprisingly, not all components of synaptic vesicles are decreased in mutant animals. Peripheral membrane proteins such as Rab3A and raphilin-3A show no statistically significant reduction, and Rab5 is even increased (Table 2). In single knockouts, the synapsin isoform that was not deleted was also decreased, suggesting that



both synapsins behave like intrinsic membrane proteins. Quantification of the messenger RNA levels of selected intrinsic membrane proteins showed no decreases (data not shown), indicating that the decrease in synaptic vesicle proteins is due to a premature degradation of these proteins in the mutant mice. This degradation appears to occur in nerve terminals as no increase in synaptic vesicle proteins could be detected in the cell bodies of the mutant mice (data not shown).

Synaptic transmission was studied in wild-type and knockout mice by extracellular recordings from CA1 pyramidal cells in hippocampal slices. Experiments on synapsin-I knockouts confirmed earlier results⁴ of increased paired pulse facilitation (PPF) but apparently normal post-tetanic potential (PTP) (Fig. 2*a, d*). Surprisingly, synapsin-II knockouts exhibited a different phenotype: PPF was normal but the magnitude of PTP was decreased (Fig. 2*b, e*). PTP was even more severely depressed in the double knockouts which, however, showed no consistent change in PPF (Fig. 2*c, f*). These data suggest that both synapsins contribute to short-term synaptic plasticity, with synapsin I acting in PPF while both synapsins contribute to PTP. Long-term potentiation could be elicited in slices ($n=4$) from double-knockout mice, suggesting that synapsins are not essential for this form of long-term synaptic plasticity (data not shown).

To achieve a more global assessment of the synaptic vesicle cycle, we used a protocol of repetitive stimulation at physiological frequencies (10 Hz for 30 s) similar to that developed to evaluate synaptic vesicle recruitment in *rab3A*-deficient mice¹³. In wild-type and synapsin-I-deficient synapses, an initial enhancement of neurotransmitter release (corresponding to augmentation/PTP¹⁴) was followed by a continuous decline, leading to synaptic depression (Fig. 2*g*). In contrast, synapses lacking synapsin II (Fig. 2*h*) or both synapsins (Fig. 2*i*) exhibited a rapid and pronounced activity-dependent depression. To test if double knockouts are subject to greater synaptic depression than synapses lacking only synapsin II, slices from wild-type mice, synapsin-II knockouts and double knockouts were analysed simultaneously (Fig. 2*j*). As observed with PTP, a larger effect was observed in the double knockouts than in synapsin-II knockouts alone. This pronounced synaptic depression cannot be attributed to differences in postsynaptic depolarization during the 10 Hz stimulation as it was also observed in whole-cell voltage clamp recordings (Fig. 2*k*).

To examine the early time course of use-dependent synaptic depression, we decreased the probability of release by lowering the extracellular Ca^{2+} concentration to 1.3 mM. Under these conditions wild-type synapses exhibited no significant depression, but synapses lacking both synapsins showed an almost immediate decrease in transmitter release, suggesting that the inability of mutant synapses to adjust to higher release rates occurs after only a few stimuli. One possible mechanism for the decrease in synaptic vesicles and the impaired transmitter release during repetitive stimulation is an increased frequency of spontaneous exocytosis leading to tonic depletion of vesicles. To test this idea, spontaneous excitatory postsynaptic currents and miniature postsynaptic currents were measured. No significant differences in the mean frequency of these events were found (data not shown). Stimulation of release by high sucrose also failed to reveal a difference between wild-type and double-knockout synapses.

In summary, mice lacking synapsins are viable but suffer from impaired presynaptic functions and a high incidence of seizures. Electrophysiologically, synapsin-II knockouts and double knockouts exhibit reduced PTP and synaptic depression upon repetitive stimulation. In contrast, synapsin-I knockouts only experience an increase in PPF but no change in PTP or repetitive release. Biochemically, levels of synaptic vesicle proteins are slightly decreased in individual knockouts and significantly decreased in double knockouts, and vesicle numbers are reduced by 50% in the double knockouts. Thus synapsins are not required for neurite outgrowth or synaptogenesis and have no essential

TABLE 2 Levels of synaptic proteins in synapsin-knockout mice

	Wild type	SynI knockout	SynII knockout	Double knockout
Synaptotagmin I	100 $\pm$ 4.8%	79.3 $\pm$ 4.0%	87.9 $\pm$ 2.6%	66.5 $\pm$ 3.9%
Synaptotagmin II	100 $\pm$ 4.7%	78.7 $\pm$ 3.3%	87.6 $\pm$ 3.5%	69.8 $\pm$ 3.5%
Synaptophysin I	100 $\pm$ 3.6%	72.9 $\pm$ 3.2%	83.6 $\pm$ 3.2%	71.4 $\pm$ 3.7%
Synaptoporin II	100 $\pm$ 3.8%	79.5 $\pm$ 4.4%	92.1 $\pm$ 2.9%	64.4 $\pm$ 2.4%
Synaptobrevin II	100 $\pm$ 5.8%	73.1 $\pm$ 3.8%	82.2 $\pm$ 4.1%	62.2 $\pm$ 4.9%
SV2	100 $\pm$ 1.8%	80.9 $\pm$ 1.6%	81.7 $\pm$ 1.5%	73.3 $\pm$ 2.2%
CaMK II	100 $\pm$ 3.3%	93.2 $\pm$ 2.5%	90.7 $\pm$ 2.1%	86.9 $\pm$ 4.1%
GDI	100 $\pm$ 3.7%	93.1 $\pm$ 2.4%	95.0 $\pm$ 2.5%	89.0 $\pm$ 3.5%
Rabphilin-3A	100 $\pm$ 3.4%	93.7 $\pm$ 2.5%	93.1 $\pm$ 2.7%	86.0 $\pm$ 3.1%
Rab3a	100 $\pm$ 2.9%	95.2 $\pm$ 2.8%	106.7 $\pm$ 2.1%	98.3 $\pm$ 2.4%
Rab4	100 $\pm$ 7.4%	100.6 $\pm$ 6.8%	108.0 $\pm$ 4.6%	115.5 $\pm$ 5.9%
Rab5	100 $\pm$ 3.7%	126.5 $\pm$ 3.6%	130.2 $\pm$ 2.7%	122.4 $\pm$ 3.5%
Dynamin I	100 $\pm$ 3.5%	109.8 $\pm$ 2.9%	110.8 $\pm$ 2.2%	103.6 $\pm$ 2.3%
Syntaxin	100 $\pm$ 4.8%	113.4 $\pm$ 4.0%	105.8 $\pm$ 3.2%	103.0 $\pm$ 4.6%
SNAP-25	100 $\pm$ 4.4%	114.1 $\pm$ 3.3%	109.1 $\pm$ 4.0%	118.4 $\pm$ 3.9%
Munc18	100 $\pm$ 3.8%	105.3 $\pm$ 2.7%	108.2 $\pm$ 3.1%	101.0 $\pm$ 4.5%
GAD	100 $\pm$ 3.8%	107.0 $\pm$ 4.8%	89.6 $\pm$ 3.0%	97.3 $\pm$ 5.9%
Synapsin I	100 $\pm$ 2.5%	—	74.4 $\pm$ 4.1%	—
Synapsin II	100 $\pm$ 5.8%	71.9 $\pm$ 6.8%	—	—

Relative levels of synaptic proteins were determined by quantitative immunoblots on samples from total brain from mice of the indicated genotypes (synI, synapsin I; synII, synapsin II). Values with a significant difference ($P < 0.01$) between wild-type and knockout are shown in bold typeface. Samples from 8 animals were analysed on each blot to control for inter-gel variability (2 independent lines for control, synI-, synII- and double-knockout mice). Blots were reacted with mono- and polyclonal antibodies against the indicated proteins^{3,4,13,21}. Signals were visualized using ¹²⁵I-labelled secondary antibodies and quantified on a phosphorimager. The signals on all immunoblots were normalized for the signal obtained with an NMDA-R1 antibody as an internal control for interlane variability and protein load. Data shown are means $\pm$ s.e.m. from a total of 24 determinations (4 independent sets of blots each for 6 animals for each genotype). All statistical analyses were performed on raw data by an ANOVA test with subsamples²⁰. The small changes in the levels of intrinsic synaptic vesicle membrane proteins observed here were not detected in the original description of the synapsin-I knockout⁴ because the small size of the change required a larger sample size than previously analysed in order to reach statistical significance.

function in the basic mechanics of the synaptic vesicle cycle. However, they appear to be selectively required for the acceleration of synaptic vesicle traffic during repetitive stimulation at physiological frequencies. Synaptic depression during repetitive stimulation may contribute to the seizure development as inhibitory GABAergic interneurons fire at high frequencies¹⁵ and may make GABA release particularly sensitive to the deletion of synapsins.

Based on these results, we propose that synapsins perform a regulatory function in increasing the supply of fusion-competent synaptic vesicles at the active zone during conditions of accelerated vesicle traffic. In the absence of this function, synaptic vesicles become partially unstable and partly degraded. Synapsin action in accelerating vesicle traffic could occur either in the movement of vesicles to the active zone, their docking at the active zone, or their maturation after docking to a fusion-competent state. The site of action and the biochemical activity of synapsins in accelerating vesicle traffic are unknown, but could involve interactions of synapsins with the actin-, tubulin- or neurofilament-based cytoskeleton^{6,10}. However, the lack of a redistribution of synaptic vesicles in the double knockouts suggests that the anchorage of synaptic vesicles on the cytoskeleton is not a primary function of synapsins. Four findings point to a post-docking role of synapsins without conclusively identifying the site of action. (1) Rab3A dissociates from synaptic vesicles in the nerve terminal after docking and is not decreased in the knockouts, indicating that destabilization of the vesicles in the knockouts occurs after docking. (2) In the knockouts, Rab5 is significantly increased (Table 1). Rab5 presumably functions on the endocytic limb of the pathway, suggesting that there may be a compensatory increase in endocytic activity which would also be post-docking. (3) The increase in PPF in the synapsin-I knockout must operate at the active zone because of its

timescale¹⁴. (4) The major difference between conventional and ribbon synapses is that ribbon synapses have a specialized docking mechanism. Ribbon synapses selectively lack synapsins¹⁶ but are not functionally altered by expression of synapsins¹⁷, suggesting that they do not need synapsins because they have developed a different mechanism for the step in which synapsins normally function.

What is the functional relationship between synapsins I and II? Individually, each synapsin knockout shows a rather low seizure incidence and small reduction in synaptic vesicle proteins. Together in the double knockout, these phenotypes are greatly enhanced, suggesting partially redundant functions for synapsins I and II in agreement with their sequence homology³. The electrophysiological phenotype of the synapsin I knockout is different from that of the synapsin II or the double knockout, however, even though the absence of synapsin I contributes to

the impairment of transmitter release in the double knockout. A definitive explanation for this apparent paradox is lacking. Synapsin I may have dual inhibitory and facilitatory functions in accelerating synaptic vesicle traffic, whereas synapsin II only has a facilitatory function. The inhibitory function of synapsin I would begin to operate on a millisecond timescale and could thus explain the enhanced PPF in the synapsin-I knockout. The facilitatory functions of synapsins I and II would be similar in operating on a timescale of seconds. The structures of the synapsins are consistent with this hypothesis in that the shared N-terminal domains, which attach synapsins to vesicles, could account for their redundant functions, and the unique negative regulation of transmitter release by synapsin I could be due to its C-terminal domain. Experiments focusing on the shared and unique phosphorylation sites in synapsins I and II will test this hypothesis. □

Received 27 January; accepted 28 April 1995.

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ACKNOWLEDGEMENTS. T.W.R., D.S. and M.M. contributed equally to this work. We thank M. S. Brown, M. Geppert, J. L. Goldstein and R. Nicoll for advice; N. Brose, P. Celio, R. Jahn, C. Li, H. McMahon, I. Mellman, M. Schachner and H. Schulman for antibodies; and L. Lundquist, I. Leznicki and A. Roth for technical assistance. This study was supported by grants from the W. M. Keck Foundation, the Perot Family Foundation (to T.C.S.), the Public Health Service (to R.C.M.), and the McKnight Endowment Fund for Neuroscience (to R.C.M.). T.R. and M.M. were partially supported by postdoctoral fellowship grants from the DFG, D.S. by training grants from the Public Health Service, and D.K.S. by a HHMI medical student fellowship.

Distinct pools of synaptic vesicles in neurotransmitter release

Vincent A. Pieribone^{*}||, Oleg Shupliakov[†],
Lennart Brodin[†], Sabine Hilfiker-Rothenfluh^{*},
Andrew J. Czernik^{*} & Paul Greengard^{*}

^{*} Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

[†] Department of Neuroscience, Karolinska Institutet, S-17177 Stockholm, Sweden

|| To whom correspondence should be addressed

NERVE terminals are unique among cellular secretory systems in that they can sustain vesicular release at a high rate. Although little is known about the mechanisms that account for the distinctive features of neurotransmitter release, it can be assumed that neuron-specific proteins are involved. One such protein family, the synapsins, are believed to regulate neurotransmitter release through phosphorylation-dependent interactions with synaptic vesicles and cytoskeletal elements¹. Here we show that clusters of vesicles at synaptic release sites are composed of two pools, a distal pool containing synapsin and a proximal pool devoid of synapsin and located adjacent to the presynaptic membrane. Presynaptic injection of synapsin antibodies resulted in the loss of the distal pool, without any apparent effect on the proximal pool. Depletion of this distal pool was associated with a marked depression of neurotransmitter release evoked by high-frequency (18–20 Hz) but not by low-frequency (0.2 Hz) stimulation. Thus the availability of the synapsin-associated pool of vesicles seems to be required to sustain release of neurotransmitter in response to high-frequency bursts of impulses.

Fluorescence-tagged synapsin antibodies, which recognize a synapsin-Ia-like protein in lamprey CNS (Fig. 1*a, b, g, h, i*), were injected into living lamprey reticulospinal axons², in which *en passant* synapses are distributed along a single main axonal trunk. Within minutes, the fluorescence became concentrated at spots corresponding to the location of synaptic active zones². This accumulation was blocked by preabsorption of antibodies with the immunogen (Fig. 1*c*). Electron microscopy revealed that synapsin antibodies caused a marked alteration in the organization of the presynaptic region. Following light stimulation (Fig. 2 legend), synapses from uninjected axons always contained large, densely packed vesicle clusters^{3,4} (Fig. 2*a*), whereas synapses from antibody-injected axons showed a dramatic loss of synaptic vesicles (Fig. 2*b*). All of these synapses (*n* = 15; Fig. 3*d*) were devoid of the outer portion of the vesicle cluster (Fig. 2*b*). The remaining vesicles were localized within 300 nm of the plasma membrane (Figs 2*b* and 3*d*). The appearance of the proximal part of the vesicle cluster remained unchanged after prolonged exposure to the antibodies (up to 3 hours). In some cases, free synaptic vesicles and endosomal-like structures were present around depleted vesicle clusters, but neither invaginations in the presynaptic membrane nor excess coated structures were observed, suggesting that endocytic retrieval of vesicle membrane was not impaired^{3,5}.

In control experiments, irrelevant antibodies (rabbit anti-mouse IgG) were injected. These antibodies failed to accumulate at spots in living axons (Fig. 1*d*), and had no effect on synaptic organization (Fig. 2*d*). As another control, antibodies to the synaptic vesicle protein synaptotagmin^{6,8}, which detected a single band in lamprey CNS (Fig. 1*f*), were injected (*n* = 8). These antibodies produced concentrated spots of fluorescence (Fig. 1*e*), identical to those obtained with the synapsin antibodies. In this case, however, the synapses showed a different type of ultrastructural change. There invariably remained a cluster of densely packed vesicles at a distance from the plasma

membrane. In contrast, the region of the cluster adjacent to the active zone often showed a reduced number of vesicles and a distorted pattern (Fig. 2c). Moreover, invaginations in the presynaptic plasma membrane and divisions in the active zone were observed, together with an increased number of endosomal-like and coated vesicular structures. These morphological changes resembled those apparent in axons fixed during conditions of intense neurotransmitter release, when the rate of vesicle fusion appears to exceed that of membrane retrieval^{3,5} (compare ref. 8).

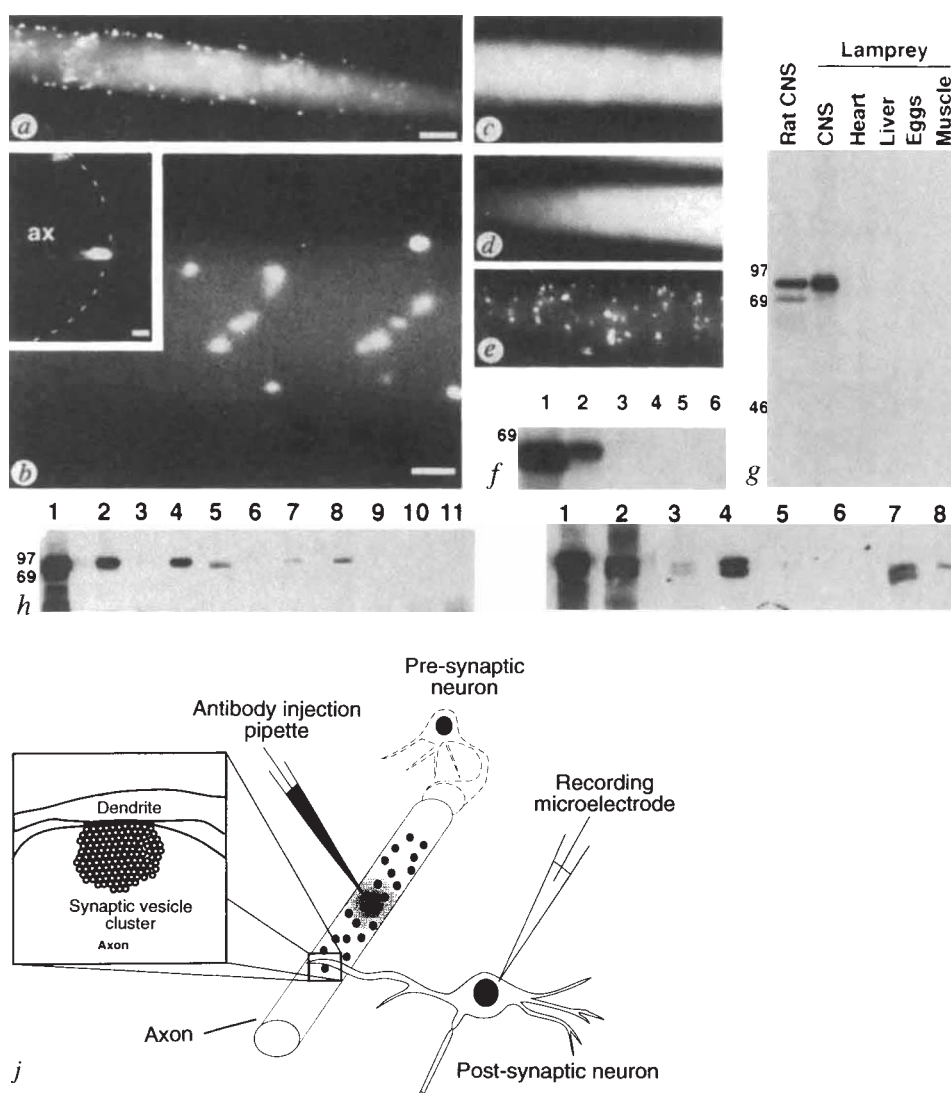
These findings suggested that synapsin contributes to the stability of a major portion of the synaptic vesicle cluster, but not to the subpopulation adjacent to the plasma membrane. Consistent with this interpretation, immunoelectron microscopic analysis of control synapses, using the same synapsin antibodies (G-304), revealed that synapsin is predominantly associated with the outer portion of the vesicle cluster, whereas vesicles in close proximity to the presynaptic membrane showed little or no label-

ling (Fig. 3a-c). Thus, the ability of synapsin antibodies to deplete the distal portion of the vesicle cluster selectively correlates with the differential distribution of synapsin in that pool.

To examine the consequences of removing the major, synapsin-associated portion of the synaptic vesicle cluster on synaptic transmission, simultaneous intracellular recordings were made from a presynaptic axon and a single postsynaptic target neuron (Figs 1j, 4). When the presynaptic axon was stimulated at low frequency (0.2 Hz), little or no change was observed in the amplitude of the excitatory postsynaptic potential (e.p.s.p.) either before or after the injection of antibodies (Fig. 4a, d). But when high-frequency stimulation (18 Hz) was applied before injection, only a moderate decrease of the e.p.s.p. amplitude^{2,9} (Fig. 4b, c) was seen, whereas such stimulation after antibody injection was associated with a pronounced decrease (Fig. 4e, f). To test if this suppression of high-frequency release could be attributable to a depletion of the small, synapsin-independent

FIG. 1 a, Injection of Cy5-labelled synapsin antibodies (G304) into lamprey reticulospinal axons resulted in a concentration of fluorescence in spots. b, High-magnification image of an axon injected with synapsin antibodies. Inset: Cross-sectional confocal image of an injected axon (ax) reveals that spots are localized to the inner face of the axonal membrane (stippled line). Injection of immunogen preabsorbed synapsin antibodies (c), or of rabbit anti-mouse antibodies (d), produced only diffusely distributed fluorescence. e, Synaptotagmin antibodies also produced fluorescent spots. f, Synaptotagmin antibodies detected a single band in rat CNS (lane 1; 100 µg of protein) and lamprey CNS (lane 2; 100 µg), but not in lamprey heart (lane 3), liver (lane 4), egg (lane 5) or muscle (lane 6). g, Tissue-specific localization of the lamprey synapsin-like protein. Immunoblot with G-304 (1:10,000 dilution) detected synapsins Ia and IIa in extracts (50 µg protein) from rat cerebral cortex (rat CNS) and one major protein in extracts (100 µg) from lamprey CNS, but not from peripheral tissues (100 µg). h, i, Crossreactivity of the lamprey synapsin-like protein with additional synapsin antibodies. Lamprey CNS extracts (100 µg) were immunoprecipitated with h, G-304, or i, G-486, and the recovered protein was subjected to SDS-PAGE and immunoblotting with a panel of antibodies having differential specificity for mammalian synapsin isoforms. Lanes: 1, G-304 (specific for Ia/IIa); 2, G-486 (Ia/b); 3, G-454/455 (Ia/b); 4-7, G-143, G357, G-299/300, G-472, respectively (all 4 isoforms); 8, G-306 (Ia); 9, G-278 (Ib); 10, G-281 (IIa); 11, G-466/467 (IIb). Thus, the lamprey protein most closely resembles synapsin Ia. j, Schematic diagram of the experimental paradigm. Calibration bars: a, c, d, e, 25 µm; b, 10 µm; inset, 2 µm. Size markers in f, g, h and i are indicated (relative molecular masses in thousands).

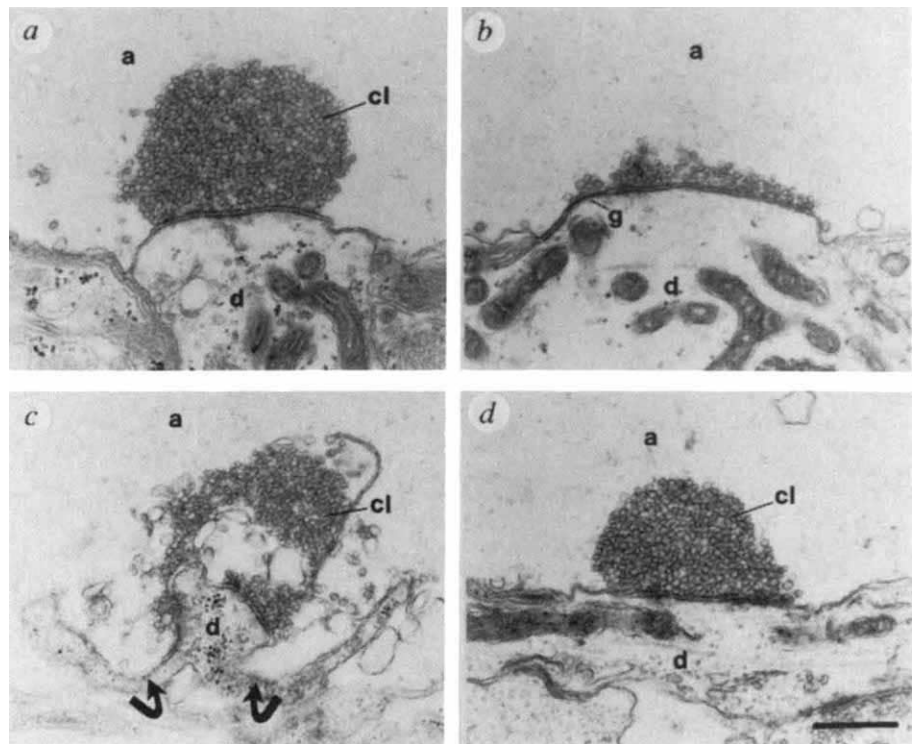
METHODS. Affinity-purified samples of G-304 (refs 17, 21) were labelled with Cy5. Synaptotagmin antibodies were raised against a recombinant polypeptide corresponding to the cytoplasmic domain of rat synaptotagmin I. Lamprey (*Lampetra fluviatilis*) spinal cords were maintained *in vitro* as described²³. Fluorescent tagged antibodies (0.6-1 mg ml⁻¹ in 180 mM potassium acetate and 10 mM HEPES, pH 7.4) were pressure-injected into reticulospinal axons² ($n > 30$; resting membrane



potential and action potential of at least -60 mV and 70 mV, respectively), and their spread was monitored with a CCD detector (Princeton Instruments). Confocal images were made after methanol fixation and incubation with FITC-coupled anti-rabbit antibodies. Antibody production, affinity-purification and immunoblotting procedures^{17,21} and immunoprecipitation²² (substituting protein A-Sepharose for pansorbin) were carried out as described. Affinity-purified antibodies were used for all experiments.

FIG. 2 Disruption of synaptic vesicle clusters in living synapses by synapsin antibody injection. Axons were injected with *a*, no antibodies; *b*, synapsin antibodies (G-304); *c*, synaptotagmin antibodies; and *d*, rabbit anti-mouse IgG antibodies. All electron micrographs correspond to the centre section from serially sectioned synapses located within 100 μm of the injection site. *a*, Axoplasmic matrix; *cl*, synaptic vesicle cluster; *d*, post-synaptic dendrite; *g*, gap junction. Arrows, invaginations in the axolemma. Scale bar, 0.5 μm . The persistence of antibody-linked fluorescence at active zones (Fig. 1) after the vesicle cluster had been disrupted may be explained by synapsin-anti-synapsin antibody complexes remaining associated with cytoskeletal and membrane components.

METHODS. Spinal cords were fixed, embedded and analysed as described²³. Axons were stimulated²³ at 1 Hz for 12 min and maintained at rest for 1.5 h before fixation. In those axons which had been injected with synapsin antibodies, all synapses examined following stimulation/rest showed a complete disappearance of the distal region of the vesicle cluster. In the absence of stimulation, the effect of antibody injection ranged from partial to complete disruption. For this reason, quantitation (Fig. 3) was carried out only on axons that had been stimulated and allowed to rest. Axons were allowed to rest following stimulation to ensure a complete return to a resting condition before fixation. The levels of stimulation had no effect on control synapses, that had not been exposed to synapsin antibodies, or on synapses exposed to rabbit anti-mouse IgG or synaptotagmin antibodies. Antibody-exposed synapses were directly



compared with control synapses from the same axon. The control synapses were examined in a region 18–20 mm caudal from the antibody injection site, at a distance well beyond the spread of the injected antibody (1–2 mm).

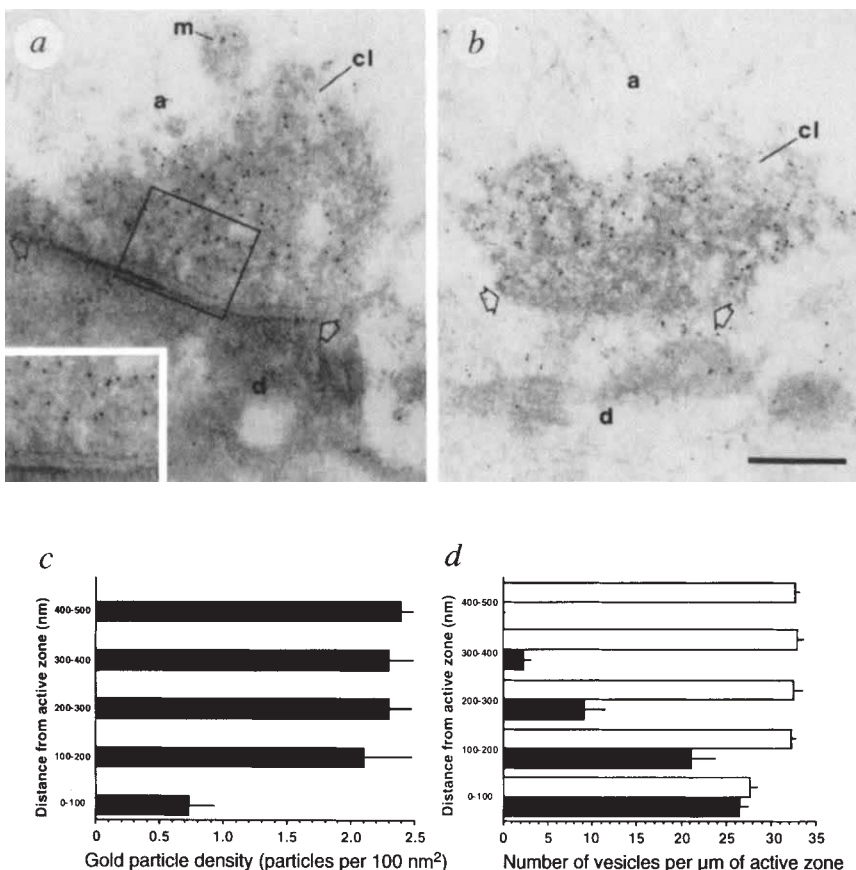
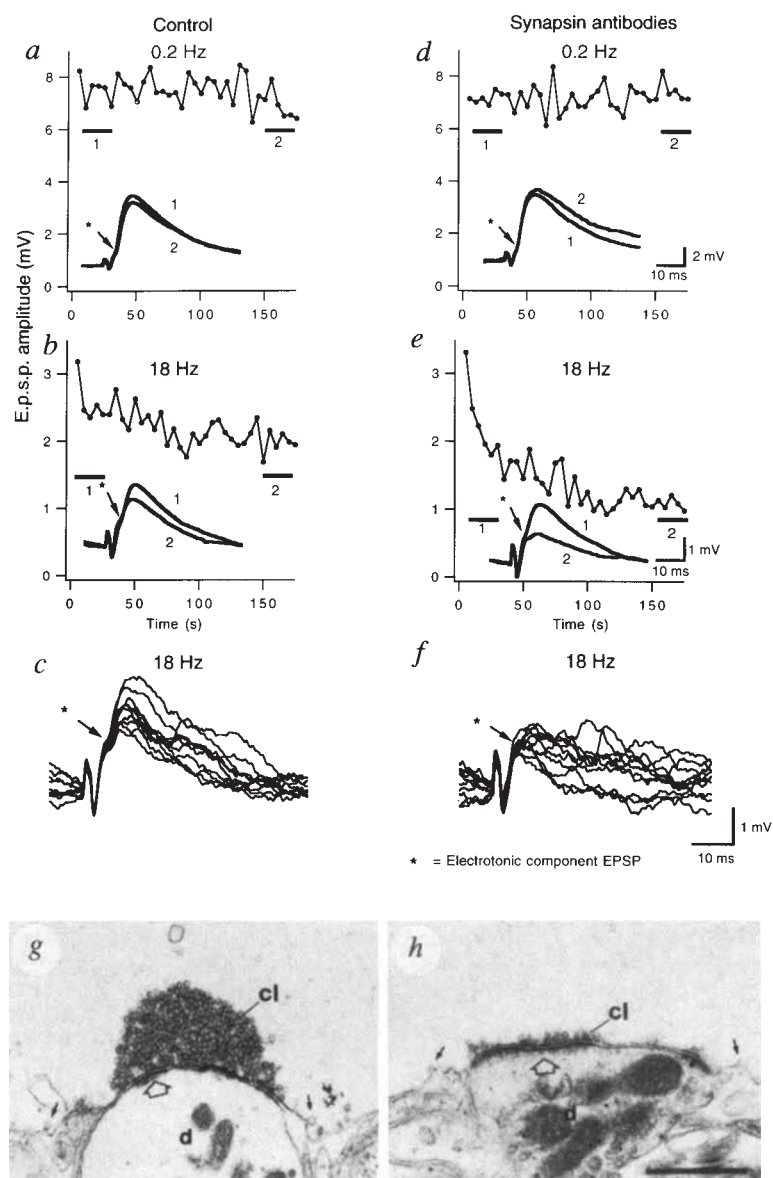


FIG. 3 Synapsin is predominantly associated with the outer portion of the synaptic vesicle cluster. *a* and *b*, Electronmicrographs of reticulospinal synapses labelled with synapsin antibody G-304 using the post-embedding immunogold method. Inset in *a* shows the framed area at high magnification. Note that the vesicles adjacent to the presynaptic plasma membrane are almost devoid of gold particles. With the post-embedding method, epitopes are equally accessible across the entire section. *m*, Mitochondrion; *cl*, synaptic vesicle cluster; *d*, postsynaptic dendrite. Open arrows indicate margins of active zones. Scale bar, 0.5 μm . *c*, Distribution of synapsin immunogold labelling in the presynaptic area of normal synapses. The diagram shows the number of gold particles per 100 nm² (mean $\pm$ s.e.m.) at varying distances from the presynaptic membrane (one synapse from each of 14 axons). Densely packed synaptic vesicles were present throughout the area included in the analysis. Few gold particles were present with 100 nm of the presynaptic membrane. Quantification was only carried out on synapses where the presynaptic membrane was clearly delineated. *d*, Loss of synaptic vesicles after injection of synapsin antibodies. Filled bars represent the number of synaptic vesicles per μm of active zone for axons injected with synapsin antibodies; open bars represent values for control axons (15 synapses from 7 axons in each case; mean $\pm$ s.e.m.). In the uninjected synapses, vesicle clusters always extended outside this range (compare Fig. 2a).

METHODS. Spinal cords were fixed in 0.5% glutaraldehyde plus 3% paraformaldehyde, dehydrated in methanol and embedded at low temperature in Lowicryl. Ultrathin sections were incubated with synapsin antibodies (G-304), followed by secondary antibodies conjugated to 5-nm gold particles and counterstained with uranylacetate.

FIG. 4 E.p.s.ps recorded from a postsynaptic neuron before and after presynaptic injection of synapsin antibodies. Control responses (a–c) were recorded during axonal stimulation at low (a, 0.2 Hz) and high (b and c, 18 Hz) rates. After injection, the arrival of synapsin antibodies (G-304) at the synaptic region was monitored visually (CCD camera) and the synaptic response was again recorded (d, 0.2 Hz; e, f, 18 Hz). c and f, Ten sweeps from the end of the high-frequency stimulation period in b and e, respectively, are shown (marked '2' in b and e). The amplitude of the initial electrotonic component (asterisks in a–f) of the e.p.s.p.^{23,24} remained constant. In a and d, each point represents the peak amplitude of individual e.p.s.ps, and in b and e, the average of 5-s epochs. Traces in a and d are averages of 5 sweeps (0.2 Hz) during the periods indicated 1 and 2). The traces in b and e are averages of 35 sweeps (18 Hz). Every e.p.s.p. and every tenth e.p.s.p. were sampled during 0.2 and 18 Hz stimulation periods, respectively. These results are representative of each of five experiments. g and h, Changes in synaptic ultrastructure induced by high-frequency stimulation; g, a synapse in an 'uninjected' axon; h, a synapse within an axon injected with synapsin antibodies. Both axons were from the same spinal cord, which was stimulated at 18 Hz for 6 min immediately before fixation. These results were representative of 10 synapses from 6 control axons and 7 synapses from 2 injected axons. d, Post-synaptic dendrite; cl, synaptic vesicle cluster; small arrows indicate coated structures; large open arrows indicate active zones. Scale bar, 1 μ m.

METHODS. Recordings were performed as described²³. Axons were stimulated at low (0.2 Hz) and high (18–20 Hz) frequency. The distance between the injection electrode and the synaptic region was 300–400 μ m. The resting potential of the postsynaptic cell was always below -55 mV and remained within ± 3 mV.



pool of vesicles that remain after antibody injection (compare with Fig. 2b), control and antibody-injected axons were fixed immediately following high-frequency stimulation. In the stimulated control axons, synaptic vesicle clusters were still present at active zones (Fig. 4g), where signs of increased endocytic activity were apparent (Fig. 4 legend and refs 3, 5). In the antibody-injected axons, an almost complete depletion of the entire pool of synaptic vesicles was apparent (compare Figs 2b and 4h).

Whereas neurotransmitter release shares a variety of properties with generalized secretion^{10–13}, synaptic vesicle exocytosis is distinguished by unique temporal properties, fundamental to its function in mediating fast, intermittent synaptic transmission. The present results indicate that synapsin is required for the organization of the major pool of synaptic vesicles into clusters at release sites. A possible role of synapsin in synaptic vesicle clustering has also been suggested on the basis of *in vitro* studies of its interaction with phospholipid-containing vesicles¹⁴. The results of a study of mutant mice lacking synapsin I (ref. 15) showed almost no phenotypic change in a variety of structural and functional parameters examined; however, a recent independent analysis of synapsin-I-deficient mice has demonstrated a number of major deficits in neuronal phenotypes (Li *et al.*, submitted), which are consistent with the role for synapsin I pro-

posed here. The fact that virtually all fast synapses contain synapsins^{1,16,17} as well as synaptic vesicle clusters^{18–20} is consistent with our conclusion that synapsin-dependent vesicle clustering confers upon nerve terminals the ability to respond with high reproducibility during a burst of neuronal activity. □

Received 6 January; accepted 28 April 1995.

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ACKNOWLEDGEMENTS. We thank S. Grillner for support and discussions; R. H. Scheller for synaptotagmin cDNA; G. Kuzmycz for help in antibody production; K. Hulténby and F. Reinhold for advice; and J. Seelye and the Hammond Bays Biological Station for lamprey. This study was supported by the US Public Health Service and the Swedish MRC.

Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120

Michael O. Westendorp*, Rainer Frank†, Christina Ochsenbauer†, Kirstin Stricker*, Jens Dhein*, Henning Walczak*, Klaus-Michael Debatin§ & Peter H. Krammer*||

*Tumorimmunology Program and †Applied Tumor Virology, German Cancer Research Centre, D-69120 Heidelberg, Germany

‡Centre of Molecular Biology and §Children's Hospital, University of Heidelberg, 6900 Heidelberg, Germany

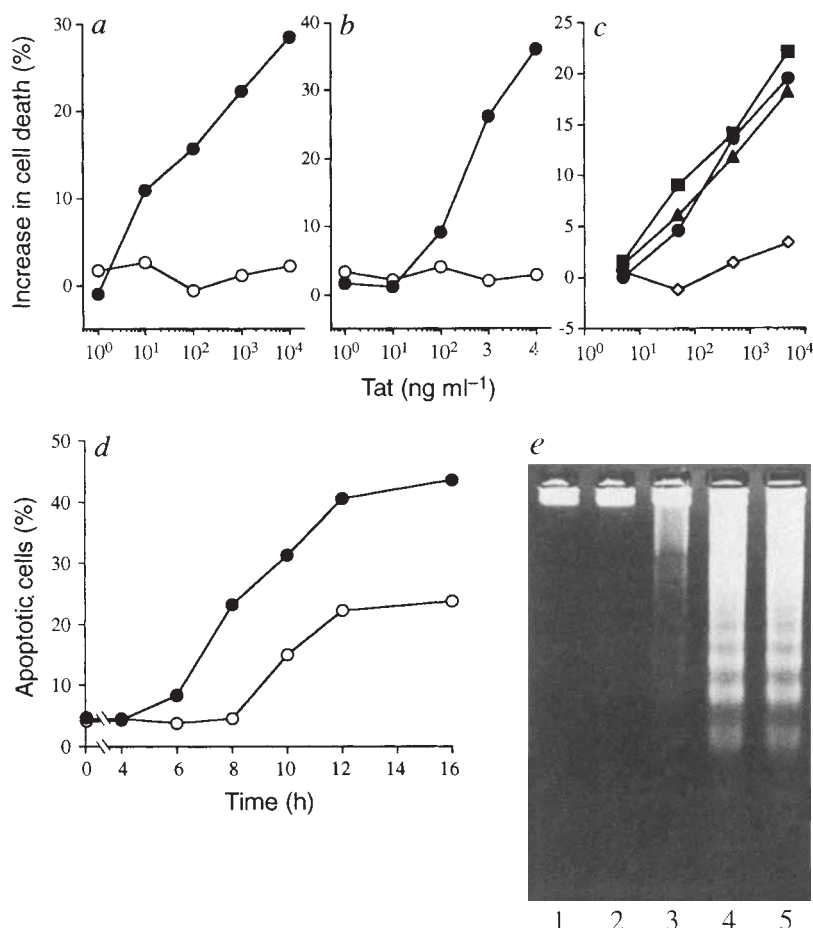
|| To whom correspondence should be addressed

THE depletion of CD4⁺ T cells in AIDS is correlated with high turnover of the human immunodeficiency virus HIV-1^{1,2} and associated with apoptosis^{3–5}. The molecular mechanism of apoptosis in HIV infection, however, is largely unknown. T-cell apoptosis might be affected by viral proteins such as HIV-1 Tat^{6–9} and gp120 (refs 10, 11). T-cell-receptor (TCR)-induced apoptosis was recently shown to involve the CD95 (APO-1/Fas) receptor¹². We show here

that HIV-1 Tat strongly sensitizes TCR- and CD4(gp120)-induced apoptosis by upregulation of CD95 ligand expression. Concentrations of Tat found to be effective in cultures of HIV-1-infected cells were also observed in sera from HIV-1-infected individuals. Taken together, our results indicate that HIV-1 Tat and gp120 accelerate CD95-mediated, activation-induced T-cell apoptosis, a mechanism that may contribute to CD4⁺ T-cell depletion^{5,13,14} in AIDS.

We investigated the effect of HIV-1 Tat on anti-CD95 and anti-CD3-induced apoptosis in Jurkat T cells, the alloreactive T-cell clone S13 (ref. 12) and in peripheral activated T cells (ATC) (Fig. 1). Tat strongly enhanced and accelerated anti-CD3- but not anti-CD95-induced apoptosis at nanomolar concentrations. As previously shown for transactivation of the HIV-1 long terminal repeat (LTR), denaturation of the protein by oxidation abolished Tat activity⁸ (Fig. 1c). Next we investigated whether sensitization of T-cell apoptosis by Tat was restricted to the CD4⁺ or CD8⁺ subset of ATC. Figure 1c shows that both subsets were equally affected. In HIV infection, however, primarily CD4⁺ T cells are progressively depleted⁵. Therefore we tested whether crosslinking of CD4, shown to induce T-cell apoptosis¹⁵, conferred T-cell subset preference to the Tat effect. Figure 2a,b shows apoptosis induced in T lymphocytes in peripheral blood mononuclear cells (PBMC). Triggering of

FIG. 1 Effect of HIV-1 Tat on anti-CD3 and anti-CD95-induced apoptosis in Jurkat T cells, the alloreactive T-cell clone S13, and ATC¹². a, b. Dose-dependent increase of anti-CD3 and anti-CD95-induced apoptosis in Jurkat and S13 cells, respectively, by Tat. Cells (5×10^5) were cultured as described¹² in the absence or presence of Tat for 12 h and subsequently distributed into 96-well-flat-bottomed plates coated with purified IgG3 anti-CD95 (anti-APO-1, 100 ng ml⁻¹) (open circles) or anti-CD3 (OKT3, 20 µg ml⁻¹) (filled circles). Cell death was assessed after a further 28 h by propidium iodide (PI) uptake ($2.5 \mu\text{g ml}^{-1}$ final concentration). Increase of cell death by Tat was calculated as percentage PI uptake in the presence of Tat minus percentage PI uptake in the absence of Tat. Anti-CD3-induced/anti-CD95-induced cell death in the absence of Tat was a, 21%/41%, and b, 44%/22%. c, Dose-dependency of the Tat-mediated increase of anti-CD3-induced apoptosis in purified CD4⁺ (filled circles), CD8⁺ (filled triangles) or mixed (filled squares) ATC by native (filled symbols) or oxidized Tat (open diamonds, mixed ATC). Anti-CD3-induced apoptosis in the absence of Tat was 27% (CD4⁺ ATC), 29% (CD8⁺ ATC) and 23% (mixed ATC). Anti-CD3-induced apoptosis was also enhanced by native Tat in the supernatant of HeLa cells stably transfected with *tat*⁷ (data not shown). d, Kinetics of anti-CD3-induced apoptosis in Jurkat T cells in the absence (open circles) or presence (filled circles) of 1 µg ml⁻¹ Tat. Tat had no effect on anti-CD95-induced apoptosis (data not shown). All assays were done in duplicate, variation was less than 12%. e, DNA fragmentation analysis¹⁷ after treatment of Jurkat T cells with medium alone (lane 1), 1 µg ml⁻¹ Tat (lane 2), 10 µg ml⁻¹ anti-CD3 (lane 3), a combination of Tat and anti-CD3 (lane 4) or 100 ng ml⁻¹ soluble anti-CD95 antibodies (lane 5). A representative experiment of three performed is shown (a–d). HIV-1 Tat was synthesized as described²⁴.



apoptosis by either the superantigen staphylococcal enterotoxin B (SEB) or crosslinking of CD4 by anti-CD4 and a combination of gp120 plus anti-gp120, respectively, was substantially increased by Tat. Tat even enhanced apoptosis in T cells induced by TCR triggering with SEB in combination with crosslinking of CD4 (Fig. 2*a, b*). Under these conditions apoptosis triggered and enhanced by the viral proteins gp120 and Tat was mainly

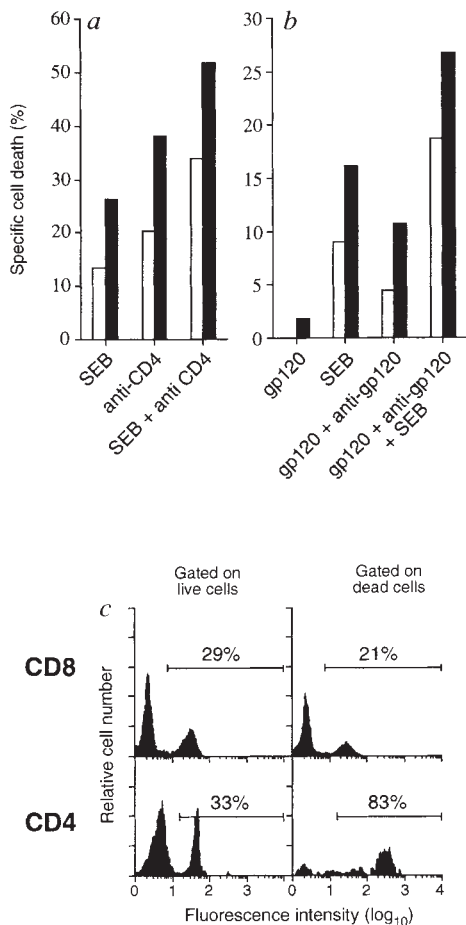


FIG. 2 Effect of HIV-1 Tat on apoptosis of PBMC induced by anti-CD4, gp120 plus anti-gp120, SEB or a combination of these reagents. *a*, Increase of specific apoptosis by Tat induced in PBMC by SEB and anti-CD4. PBMC¹⁵ were cultured in the absence (open squares) or presence (filled squares) of Tat (1 µg ml⁻¹) for up to 12 h and subsequently with monoclonal anti-CD4 antibodies (3 µg ml⁻¹) at 4 °C for 1 h and transferred into 24-well flat-bottomed plates coated with goat-anti-mouse IgG antibodies (1 µg ml⁻¹). After 3 h at 37 °C SEB (50 ng ml⁻¹) was added, and after a further 24 h apoptosis in CD4⁺ T cells was measured²⁵. The percentage of specific cell death was calculated as follows: 100 × (experimental PI uptake (%) – spontaneous PI uptake of cells in medium (%)) ÷ (100% – spontaneous PI uptake (%)). *b*, Increase of specific cell death by Tat induced in PBMC by SEB, recombinant gp120 (refs 26–29) plus anti-gp120 or a combination of these reagents. PBMC untreated (open squares) or treated with HIV-1 Tat (filled squares) as in *a* were incubated for 1 h at 4 °C with gp120 (100 ng ml⁻¹), followed by addition of polyclonal anti-gp120 (100 ng ml⁻¹). After 1 h at 37 °C SEB (50 ng ml⁻¹) was added. After 24 h specific cell death was assessed as in *a*. Anti-gp120 alone did not induce cell death above medium background levels. All assays were done in duplicate and variation was less than 11% of the mean. A representative experiment of three performed is shown. *c*, Fluorescence analysis of PBMC treated with a combination of Tat, SEB, gp120 and anti-gp120 and incubated for 24 h as described in *b*. Cells were stained using monoclonal anti-CD8 (Immunotech, Marseille, France) and polyclonal anti-CD4 (IC Chemicals, Ismaning, Germany) antibodies, respectively. Forward and side scatter analysis was used to gate on live and dead cells. The percentage of positive cells is indicated.

observed in the fraction of CD4⁺ T cells (Fig. 2*c*). Cell surface expression of CD95, TCR and CD4 was not significantly upregulated upon stimulation of ATC with Tat (data not shown), suggesting that sensitization of apoptosis was not regulated by receptor density¹⁶. Furthermore, because apoptosis induced by agonistic anti-CD95 antibodies^{17,18} was not affected by Tat (Fig. 1*a, b*), it is unlikely that Tat interferes with signals downstream of the CD95 receptor. It has previously been shown that exogenous and endogenous HIV-1 Tat shifts the redox potential of T cells towards a pro-oxidative state, activates NF-κB¹⁹ and transactivates transcription of cellular genes^{7,9}. In addition, we have shown that TCR-induced apoptosis is mediated by means of the CD95 ligand¹². Therefore we investigated the effect of HIV-1 Tat on expression of the CD95 ligand. We found that expression of CD95 ligand messenger RNA was strongly enhanced and accelerated in anti-CD3-stimulated Jurkat T cells and in anti-CD4-stimulated PBMC in the presence of HIV-1 Tat (Fig. 3*a, b*). Thus the effect of Tat on T-cell apoptosis was mediated by increased CD95 ligand expression. In agreement with this observation, sensitization of T-cell apoptosis by Tat was substantially inhibited by both F(ab')₂ anti-CD95 antibody fragments and soluble CD95 receptor decoys (CD95 immunoglobulin fusion proteins). Furthermore, a similar inhibition of gp120-induced T-cell apoptosis by these reagents was observed (Fig. 4). Moreover, the number of apoptotic cells in a low-level HIV-1-infected H9 T-cell leukaemia culture exceeded the number of productively infected cells by a factor of 4 to 10 (Fig. 4*e, f*). Thus activation-induced apoptosis occurred, at least in part, in non-infected T cells. This is in agreement with the observation that apoptosis in lymph nodes of HIV-1-infected individuals is also seen in non-infected cells⁴. Figure 4*e* also shows that activation-induced apoptosis in low-level HIV-1-infected H9 cultures was also mediated by means of the CD95 receptor/

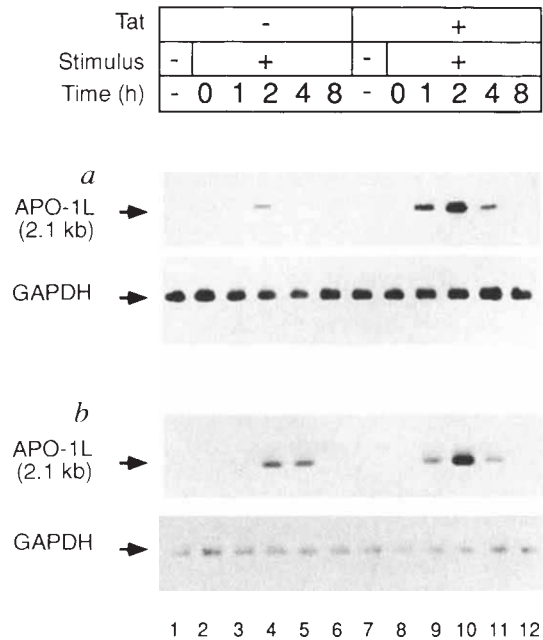


FIG. 3 Upregulation of CD95 ligand (CD95L) mRNA levels by HIV-1 Tat in *a*, anti-CD3-stimulated Jurkat cells and *b*, CD4-stimulated PBMC. *a*, Jurkat cells were either non-stimulated (lane 1) or stimulated for the indicated time with coated anti-CD3 (10 µg ml⁻¹) (lanes 2–6, 8–12) in the absence (lane 1 to 6) or presence (lane 7 to 12) of Tat (1 µg ml⁻¹). Suboptimal concentrations of anti-CD3 were used to give only minor apoptosis in the absence of Tat. *b*, PBMC were stimulated with anti-CD4 antibodies as described in Fig. 2 and Tat (1 µg ml⁻¹) as indicated. The mRNA was prepared, blotted and filters probed as described¹². A complementary DNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was used to control equal RNA loading.

CD95 ligand system, and could be blocked by $F(ab')_2$ anti-CD95 antibody fragments. In these cultures a similar blocking effect was observed by neutralizing anti-Tat antibodies (Fig. 4f). Thus native HIV-1 Tat is important in accentuated activation-induced apoptosis of HIV-1⁺ T-cell cultures. The quantity of Tat detected in some sera of HIV-1-infected individuals corresponded to the concentrations of Tat found in the supernatant of HIV-1-infected H9 cells (Fig. 4g). These data strongly suggest that effective Tat concentrations might also be reached *in vivo*, where Tat-containing non-infected and infected cells have

recently been demonstrated²⁰. High concentrations of Tat and gp120 might particularly be found in lymph nodes of HIV-1⁺ individuals, where productively infected cells are most frequent^{21,22}.

Triggering of CD4 (by gp120) or TCR (by antigen) can induce apoptosis in CD4⁺ ATC. Our findings show that in both cases apoptosis is mediated by the CD95 receptor/CD95 ligand system and is strongly sensitized by means of upregulation of expression of the lethal CD95 ligand. Tat activity might involve a shift of the cellular redox state towards pro-oxidative conditions¹⁹. Thus, in

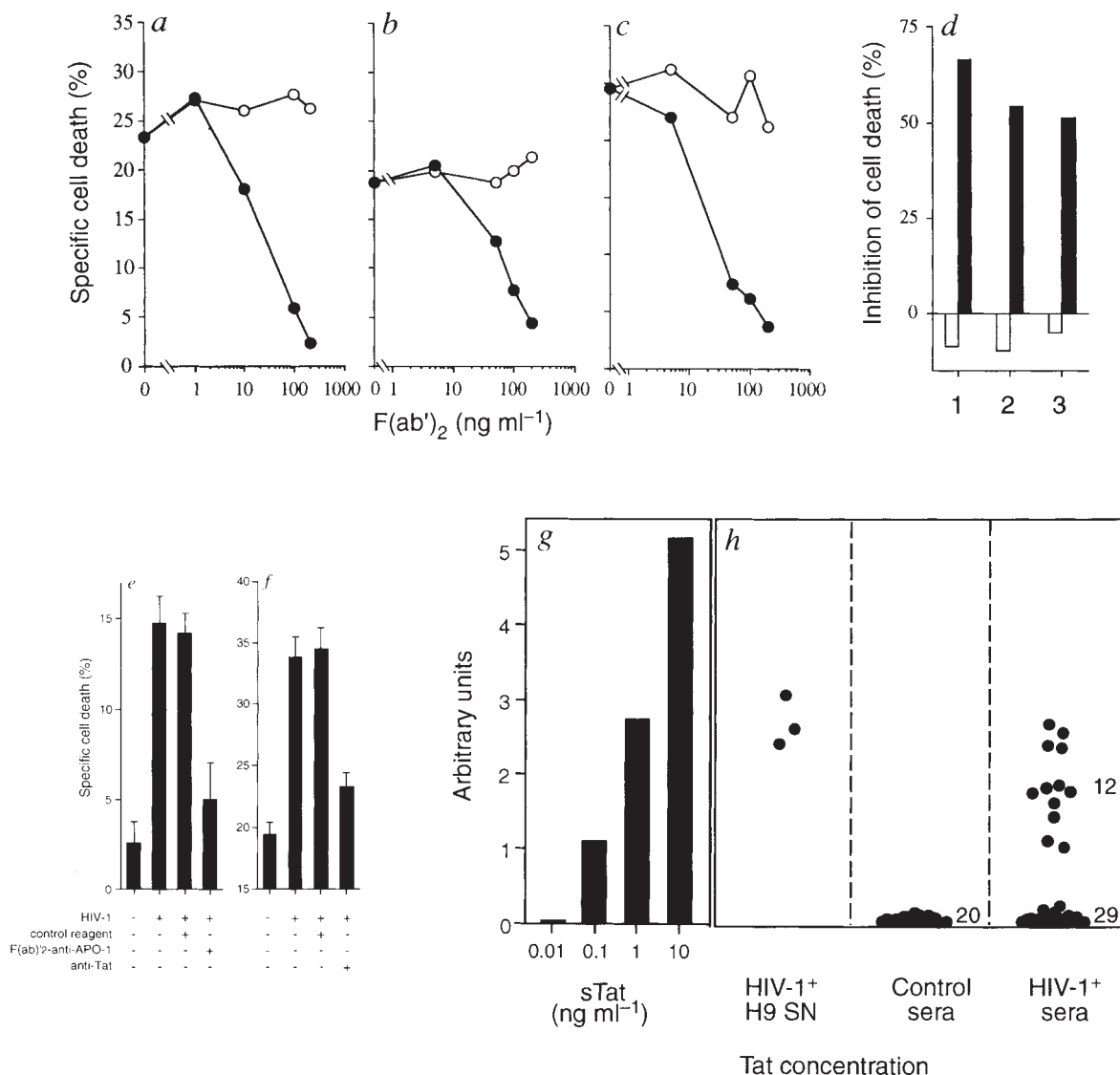


FIG. 4 Inhibition of HIV-1 Tat-sensitized TCR- and CD4-induced apoptosis (a–f) and detection of Tat in HIV-1⁺ sera (g, h). Jurkat T cells (a, d1) pretreated with Tat (1 μ g ml⁻¹) for 12 h were stimulated for 24 h by anti-CD3 (10 μ g ml⁻¹). PBMC (b, c, d2, d3) were stimulated with gp120 and anti-gp120 (b, d2) or a combination of SEB, gp120, anti-gp120 and Tat (c, d3) as described in Fig. 2. Cells were cultured in the presence of $F(ab')_2$ anti-CD95 ($F(ab')_2$ anti-APO-1)¹² (filled circles), of isotype-matched $F(ab')_2$ control antibody (open circles), CD95-immunoglobulin fusion protein (huAPO-1-Fc¹²; 20 μ g ml⁻¹; filled squares), or control IgG1 (20 μ g ml⁻¹; open squares). Anti-CD3 at the concentrations used induced only marginal kill (8%) in the absence of Tat (a). All assays were done in duplicate; variation was less than 13%. Representative experiments of three performed are shown. e, H9 T leukaemia cells were infected with supernatant containing HIV-1 (derived from pNL4-3_{BH10-env} (ref. 30)) to give a low level of productively infected cells (0.8–3.0%) detected as described³⁰. Cells were stimulated with anti-CD3 in

the presence of 100 ng ml⁻¹ $F(ab')_2$ anti-CD95 or isotype-matched $F(ab')_2$ control antibody as above, PI-stained, fixed with paraformaldehyde (10 g l⁻¹) and analysed by FACS. f, H9 cells were infected as in e in the presence of anti-Tat serum⁷ diluted 1/500 or normal rabbit serum as control. Anti-CD3-stimulated cells were analysed as above. Percentage cell death in the absence of anti-CD3 was 12% (e) and 10% (f). Mean and s.d. of one of three (e) and two (f) experiments are shown. g, h, Sera from HIV-1-infected individuals (HIV-1⁺ sera, $n=33$, HIV controls (control sera, $n=20$) and supernatant (SN) of HIV-1-infected H9 cells (see e, f) were tested for the presence of Tat. Synthetic Tat (sTat) added to a control serum was used as standard (g). SN and sera adjusted to 1% Triton X-100 and 1 M NaCl were size fractionated (M_r 5K to 30K) and concentrated. Tat was detected by ECL-Dot blot as described¹⁹ with an additional peroxidase-anti-peroxidase amplification step. Dots on exposed films were scanned densitometrically (Scan-Analysis Software, London) and arbitrary units defined.

contrast to its reverse effect on cell survival upon serum starvation²³, HIV-1 Tat accelerates the physiological process of activation-induced CD95-mediated T-cell apoptosis. The effects of Tat could occur in both infected and non-infected cells. These and other^{5,13,14} events might contribute to the depletion of CD95⁺ CD4⁺ T cells in AIDS. The molecular characterization of this process and tools to reduce increased pathological CD95-mediated apoptosis to physiological levels may allow new therapeutic interventions in AIDS. □

Received 28 February; accepted 13 April 1995.

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ACKNOWLEDGEMENTS. We thank M. Kraft for synthesis of HIV-1 Tat, V. Bosch, J. Sträter and P. Möller for discussion; S. Klevenz and G. Hölzl-Wenig for technical assistance; G. Moldenhauer for anti-CD4 antibodies; and D. Kabelitz and J. Borst for cells. The following reagent was obtained through the AIDS Reagent and Reference Reagent Program, Division of AIDS, NIAID, NIH: gp120 from HIV_{SR2} from N. Haigwood of Chiron Company. This work was supported by a grant from the Bundesministerium für Forschung und Technologie, Bonn, Germany.

Glucosylation of Rho proteins by *Clostridium difficile* toxin B

I. Just*, J. Selzer*, M. Wilm†, C. von Eichel-Streiber‡, M. Mann† & K. Aktories*

* Institut für Pharmakologie und Toxikologie der Universität des Saarlandes, D-66421 Homburg/Saar, Germany

† EMBL, D-69012 Heidelberg, Germany

‡ Institut für Medizinische Mikrobiologie der Johannes-Gutenberg-Universität, D-55101 Mainz, Germany

TOXIN A and B, the major virulence factors of *Clostridium difficile*, are the causative agents of antibiotic-associated pseudomembranous colitis. In cultured cell lines their potent cytotoxicity results from their ability to induce disaggregation of the microfilament cytoskeleton^{1,2}. Toxin B acts on the low-molecular-mass GTPase RhoA^{3,4}, which is involved in the regulation of the actin cytoskeleton. We report here that toxin B catalyses the incorporation of up to one mole of glucose per mole of RhoA at the amino acid threonine at position 37. The modification was identified and localized by tandem electrospray mass spectrometry. UDP-glucose selectively serves as cosubstrate for the monoglucosylation reaction catalysed by toxin B. Microinjection of RhoA previously glucosylated by toxin B into monolayer cells caused disaggregation of actin filaments, indicating a dominant-negative activity of glucosylated RhoA.

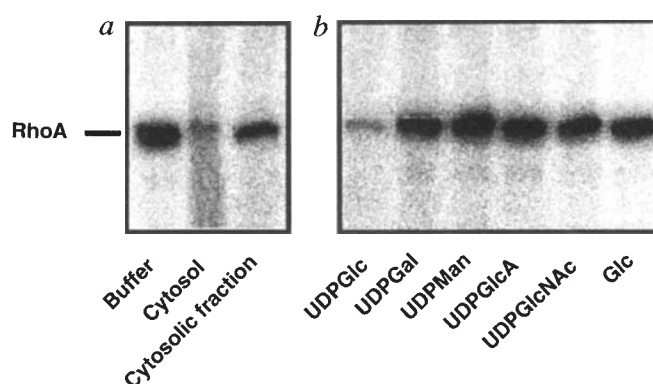


FIG. 1 ToxB-induced decrease of C3-catalysed ADP-ribosylation of RhoA. Influence of a, cytosol and b, various hexoses. RhoA was incubated with ToxB in the presence of either cytosol, a cytosolic fraction or in the presence of various UDP-hexoses followed by *C. botulinum* C3-catalysed [³²P]ADP-ribosylation.

METHODS. Recombinant RhoA (50 µg ml⁻¹) was incubated with ToxB (1 µg ml⁻¹) in the presence of either cytosol from rat basophilic leukaemia cells (50 µg ml⁻¹) or a cytosolic fraction for 60 min at 37 °C. The cytosolic fraction was prepared by incubating the cytosol for 15 min at 95 °C, followed by centrifugation and passing the supernatant through a membrane filter (cut off at *M*_r 3K). RhoA was incubated with UDP-hexoses (50 µM; Boehringer) at 37 °C for 60 min (UDPGlc, UDP-glucose; UDPGal, UDP-galactose; UDPMan, UDP-mannose; UDPGlcNAc, UDP-glucuronic acid; Glc, glucose). Thereafter, the samples were subjected to [³²P]ADP-ribosylation by C3 exoenzyme (1 µg ml⁻¹ of C3 and 10 µM of [³²P]NAD for 30 min at 37 °C). Phosphorimage data (Molecular Dynamics of the SDS-gel electrophoresis are shown. RhoA was prepared from glutathione S-transferase (GST-RhoA) fusion protein generated from a bacterial expression system (pGEX-2T vector).

C. difficile toxin B (ToxB)-induced depolymerization of actin filaments is correlated with a decrease in C3-catalysed ADP-ribosylation of Rho proteins^{3,4}. ToxB also acts in cell-free systems on recombinant Rho, but this action depends on the presence of a cellular cofactor which is heat-stable, not proteinaceous and which exhibits a relative molecular mass between 500 and 3,000 (*M*_r 0.5–3K) (Fig. 1a). Recombinant RhoA that had been modified by ToxB in a cell-free system was subjected to electrospray mass spectrometry. The *M*_r of the modified protein was 162 ± 0.5 higher than the *M*_r of unmodified protein, suggesting that toxin-modified RhoA contains a covalently bound hexose moiety (*M*_r 180 of hexose minus *M*_r 18 of released H₂O) (Fig. 2b). These data are consistent with a monoglucosylation of RhoA. To identify the acceptor amino acid of glycosylation, modified and unmodified RhoA were digested with endopeptidase Lys-C and separated by high-performance liquid chromatography (HPLC). One peptide of the digest of the modified RhoA showed a shift to earlier retention time in the HPLC separation. This peptide and its unmodified form were sequenced by electrospray mass spectrometry using a micro-electrospray source⁵. The peptide encompassed residues Asp 28 to Lys 51. Mass spectrometry of the modified peptide and of peptides generated by subdigestion with pepsin localized the modification to Thr 37 (Fig. 2). This is consistent with the observed absence of Thr 37 in automated Edman degradation of the modified peptide.

ToxB-induced decrease in C3-catalysed ADP-ribosylation of RhoA was inhibited by UTP, suggesting UDP-hexoses as the activated form of the cosubstrate. Among several UDP-hexoses, UDP-glucose strongly decreased ADP-ribosylation of Rho, whereas other UDP-hexoses and GDP-mannose, had no effect (Fig. 1b). Using radioactive UDP-[¹⁴C]glucose, ToxB catalysed labelling of recombinant RhoA in a time-dependent manner with maximal incorporation of 0.8 mol of glucose per mol of RhoA (Fig. 3a). Denaturation of RhoA or ToxB completely abolished incorporation of glucose (Fig. 3b).

Peptide Sequence: DQFPEVYVPTVFENYVADIEVDGK

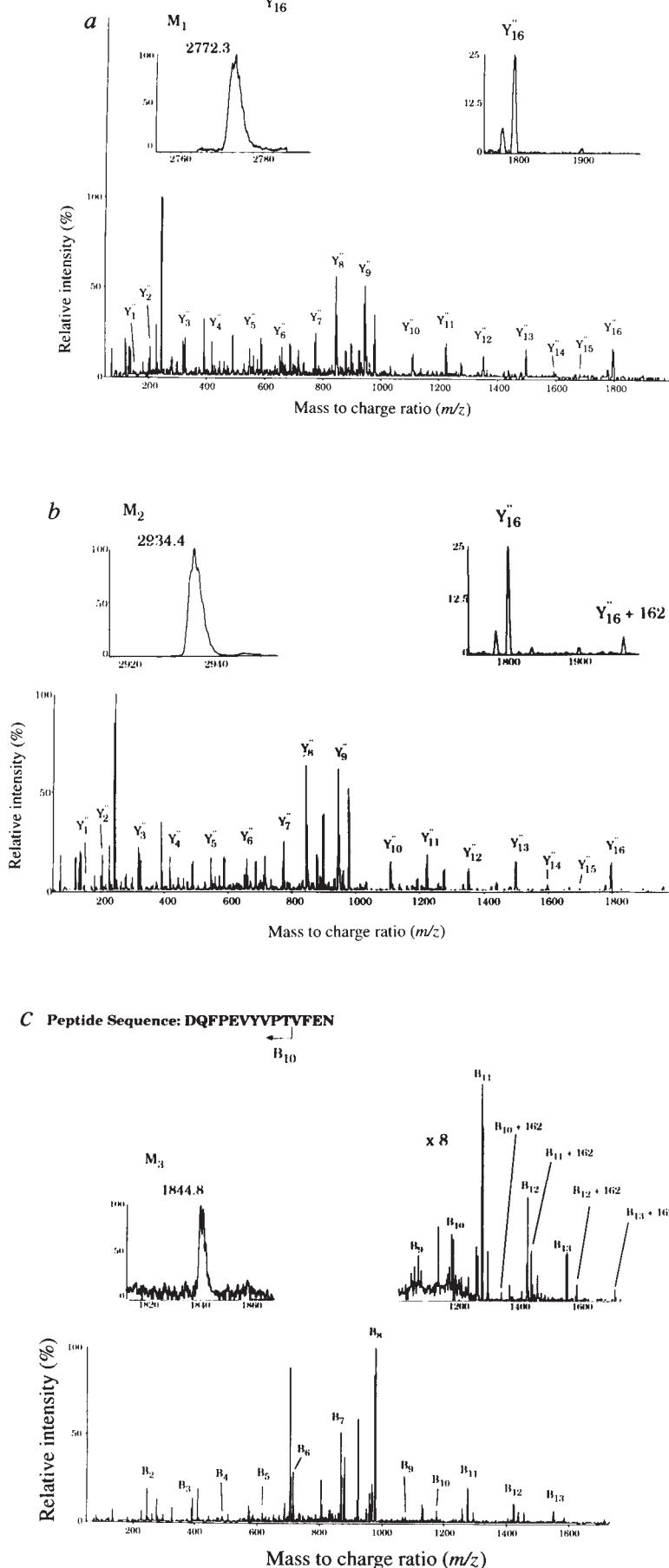


FIG. 2 Localization of the modification by tandem electrospray mass spectrometry. Mass spectrometric sequencing was performed on a Sciex API III triple quadrupole mass spectrometer (Sciex-Perkin-Elmer)¹¹. **a**, Modified and control Rho were digested with endopeptidase Lys-C (Boehringer) in the presence of urea for 16 h, followed by chromatography on Sephasil RP-C18 column with a trifluoroacetic acid (0.01%)/acetonitril gradient (up to 70%). One peptide from modified Rho showed a shift to earlier retention time. This peptide was analysed by mass spectrometry and revealed a mass which is consistent with the unmodified peptide Asp-28 to Lys-51 (D28-K51) (expected monoisotopic M_r , 2,772.3). Sequencing by electrospray mass spectrometry confirmed the sequence by a series of B and Y⁺ ions (fragments with charge retention on the amino-terminal and carboxy-terminal part, respectively, of the molecule)¹². The Y⁺ ion series covering 16 C-terminal amino acids of the peptide sequence are marked. **b**, Measurements of the modified peptide. The measured mass is consistent with a modification in M_r of 162. The same sequence-specific fragment ions were found as in **a**. This observation shows that fragmentation of the conjugate is followed by fragmentation of a peptide bond. Nevertheless, comparison of the spectra of the modified and unmodified peptide in the region around fragment ion Y⁺₁₆ (see inserts of **a** and **b**) shows an accompanying fragment ion at the modified peptide that has a M_r 162 higher. This localizes the modification to the C-terminal 16 amino acids in the peptide sequence. **c**, Tandem mass spectrometric analysis of a peptide from a pepsin subdigest of the modified peptide D28-K51. A peptide, M_r 1,844.8, was sequenced and identified as peptide D28-N41 in the modified form. A series of fragment ions for which the modification is observed localizes the modification to Thr 37. The N41-K51 peptide from the pepsin subdigest was sequenced and was without modification.

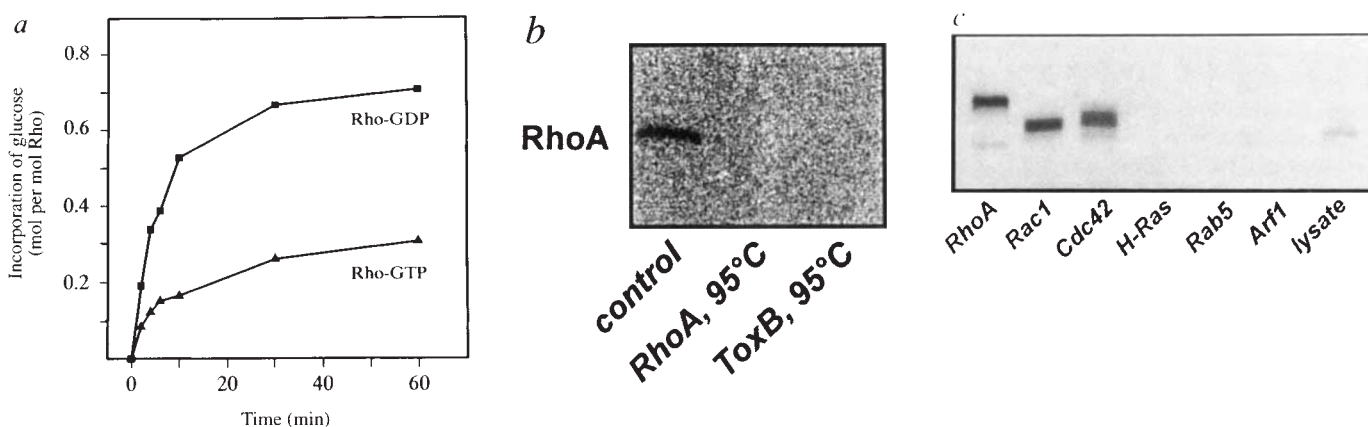


FIG. 3 ToxB-catalysed incorporation of glucose into RhoA. *a*, Time course of ToxB-catalysed incorporation of glucose into RhoA either bound to GDP (squares) or GTP (triangles). *b*, Glucosylation of either native RhoA or denatured RhoA by native or heat-inactivated ToxB. METHODS. Recombinant RhoA ($50 \mu\text{g ml}^{-1}$) was incubated with ToxB ($1 \mu\text{g ml}^{-1}$) and UDP- ^{14}C glucose ($30 \mu\text{M}$) for the indicated times at 37°C . Incorporation of glucose into RhoA was evaluated from SDS gel electrophoresis with the Phosphorimager (Molecular Dynamics). RhoA and ToxB were denatured by incubating for 15 min at 95°C , followed by an incubation in the presence of UDP- ^{14}C glucose and

either native RhoA or native ToxB for 60 min at 37°C . *c*, Substrate specificity of *C. difficile* ToxB-catalysed glucosylation. Recombinant low-molecular-mass GTPases were incubated with ToxB in the presence of UDP- ^{14}C glucose.

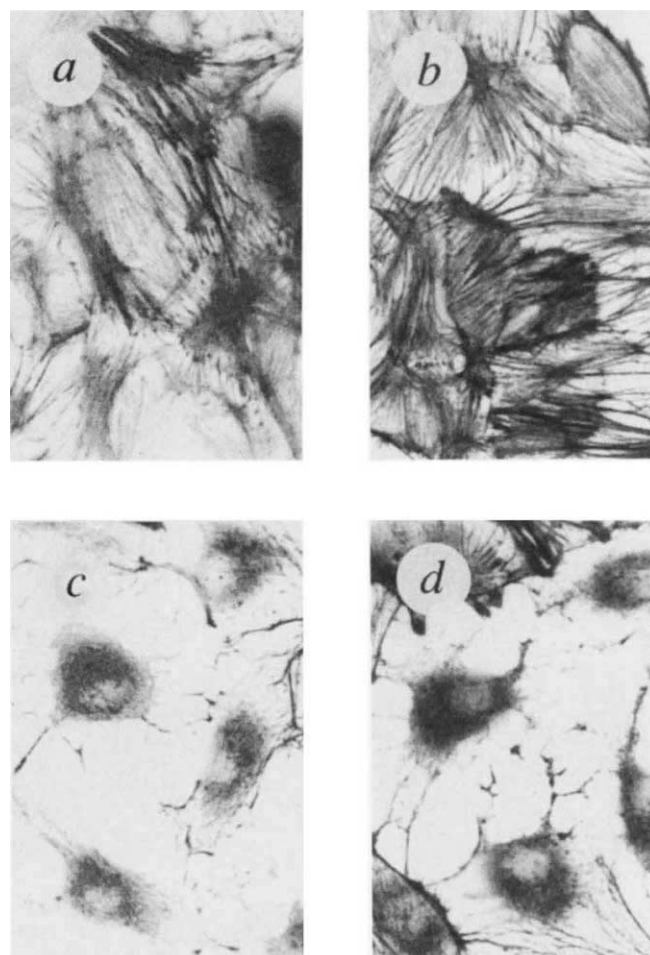
METHODS. RhoA, Rac1, Cdc42, H-Ras, Rab5, Arf1 (each $50 \mu\text{g ml}^{-1}$) (dissolved in a buffer containing 2 mM MgCl_2 , 0.3 mM GDP and 50 mM triethanolamine-HCl, pH 7.5) and rat basophilic leukaemia cell lysates (1 mg ml^{-1} protein) were incubated with ToxB ($1 \mu\text{g ml}^{-1}$) and UDP- ^{14}C glucose ($30 \mu\text{M}$) for 30 min at 37°C . Phosphorimage data of the SDS-gel electrophoresis are shown.

Incubation of cell lysates with UDP- ^{14}C glucose and ToxB resulted in labelling of proteins of M_r 20–22K only. Figure 3c shows the substrate specificity of ToxB-catalysed glucosylation. All members of the Rho family (RhoA, Rac1 and Cdc42) were glucosylated by ToxB, whereas H-Ras, Rab5 and Arf1, members of the other subfamilies of the Ras superfamily, were not substrates for ToxB. Because all the members of the Ras superfamily share a threonine residue in domain G-2 at the same position⁶, it seems that structural requirements only present in Rho proteins are essential for modification by ToxB.

As can be deduced from the crystal structure of H-Ras, Thr 37 of Rho is located in the GTP-binding and hydrolysing domain (G-2) and participates in the coordination of Mg^{2+} which is a ligand for the β - and γ -phosphates of GTP⁷. In the GDP-bound form, loop L2 moves and Thr 35 (Thr 37 in Rho) is freely exposed and accessible⁸. Consistent with this model is the finding that GDP-bound Rho is a superior substrate for ToxB to GTP-bound Rho (Fig. 3a). Furthermore, Thr 37 is located in the putative effector domain of Rho⁹, and attachment of a hydrophilic glucose group to this domain may interfere with Rho-effector coupling.

To test whether monoglucosylation of RhoA is the molecular basis of ToxB-induced cytoskeletal effects, modified RhoA was microinjected after removal of ToxB into *Potorous tridactylis* kidney epithelial (PtK2) monolayer cells, and changes in the cytoskeletal organization were monitored 45 min after microinjection. Glucosylated RhoA induced disruption of actin filaments accompanied by morphological features which are identical to those caused by microinjection of ToxB (Fig. 4).

FIG. 4 Rhodamine-phalloidin staining of the actin cytoskeleton of PtK2 cells after microinjection of glucosylated RhoA. RhoA ($400 \mu\text{g ml}^{-1}$) was incubated with ToxB ($1 \mu\text{g ml}^{-1}$) in the presence or absence of UDP-glucose ($100 \mu\text{M}$). Thereafter, ToxB (M_r 270K) was removed from a–c by centrifugation through membranes with an exclusion M_r of 100K (Microcon 100 from Amicon). The filtrates were then microinjected into PtK2 monolayer cells with a Microinjector 5242 (Eppendorf). After 45 min, cells were fixed with paraformaldehyde (4%) plus Triton X-100 (0.2%) and the actin cytoskeleton was visualized by staining with rhodamine-phalloidin. Microinjection of control RhoA had no effect on the actin filament system. *a*, UDP-glucose plus ToxB in the absence of RhoA; *b*, RhoA ($400 \mu\text{g ml}^{-1}$) treated with ToxB in the absence of UDP-glucose; *c*, RhoA ($400 \mu\text{g ml}^{-1}$) treated with ToxB plus UDP-glucose; *d*, ToxB was directly microinjected.



different from that of the double-mutant Val-14/Ala-37 Rho, in which an Ala 37 mutation blocks the activating properties of the constitutively active ^{Val14}RhoA but does not render Rho into a dominant-negative form¹⁰.

To determine whether Rho proteins are glucosylated in intact cells, we used differential glucosylation. Lysates from rat basophilic leukaemia cells, which were treated with ToxB until the cells showed typical morphology, were subjected to a second glucosylation in the presence of UDP-[¹⁴C]glucose. The radioactively labelled Rho proteins were analysed by phosphorimaging. Rho from lysates of control cells showed an incorporation of 178 ± 10 (s.e.m.) c.p.m. ($n=3$) of [¹⁴C]glucose, whereas Rho from lysates of toxin-treated cells revealed a reduced incorporation of 43 ± 7 c.p.m. ($n=3$) of [¹⁴C]glucose. The difference is significant at a level of $P<0.01$, indicating previous glucosylation of Rho proteins in the intact cell by ToxB.

In summary, we show here that the potent cytotoxin *C. difficile* ToxB is a monoglucosyltransferase which catalyses the incorporation of glucose into Thr 37 of RhoA. This modification renders Rho inactive and Rho loses its property to induce polymerization of actin filaments. Inactivation of Rho by glucosylation appears to be the molecular mechanism by which *C. difficile* ToxB mediates its cytotoxic effects in cells. Modification of regulatory proteins by monoglucosylation is a novel mechanism of intracellular acting toxins. □

Received 14 December 1994; accepted 29 March 1995.

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ACKNOWLEDGEMENTS. We thank M. Zerial for the gift of Rab5; M. Aepfelbacher for Cdc42Hs; M. Schmidt for Arf1; and M. Lerner and G. Kiefer for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16

Jiri Lukas*, David Parry†, Louise Aagaard*, David J. Mann†, Jirina Bartkova*, Michael Strauss*‡, Gordon Peters† & Jiri Bartek*§

* Danish Cancer Society, Division of Cancer Biology, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark

† Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

‡ Max-Planck-Gesellschaft, Max-Delbrück-Haus, R. Rössle Strasse 10, D-13122 Berlin-Buch, Germany

D-TYPE cyclins, in association with the cyclin-dependent kinases Cdk4 or Cdk6, promote progression through the G1 phase of the cell cycle^{1–6} by phosphorylating the retinoblastoma protein (RB)^{7,8}. The activities of Cdk4 and Cdk6 are constrained by inhibitors^{9–12} such as p16, the product of the *CDKN2* gene on human chromosome 9p21 (refs 12–14). The frequent deletion or mutation of

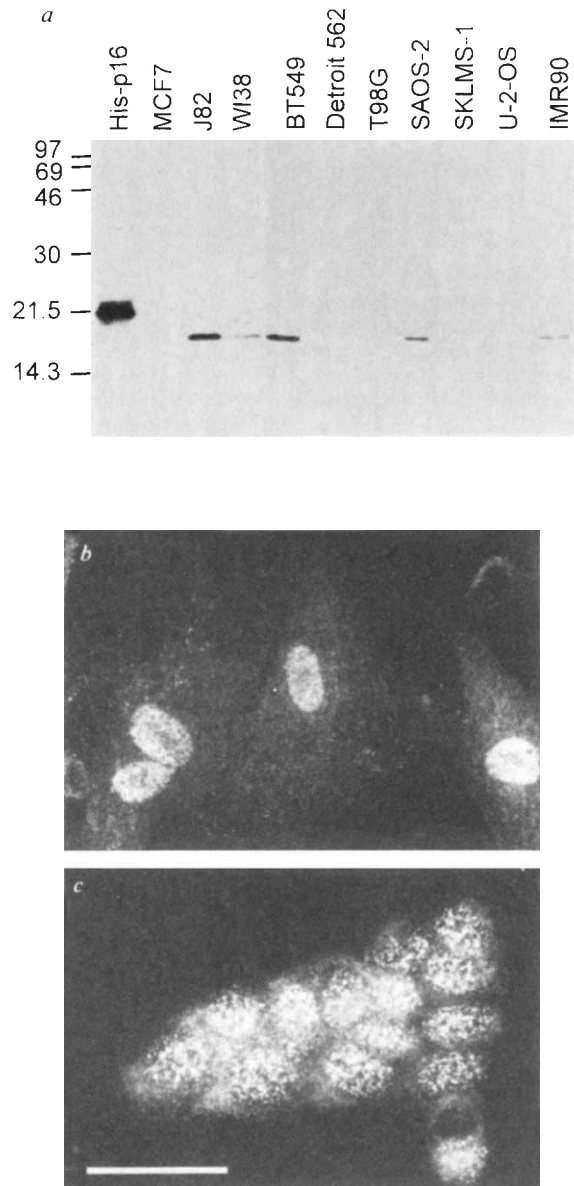


FIG. 1 Abundance and subcellular localization of endogenous p16 in tumour cell lines and normal diploid cells. *a*, Immunoblot analysis of total cell lysates shows higher levels of p16 in the RB-deficient J82 (urinary bladder carcinoma), BT549 (breast carcinoma) and Saos2 (osteosarcoma) cells as compared with WI38 and IMR90 diploid fibroblasts, and demonstrates the specificity of the monoclonal antibody DCS-50. The negative MCF-7 (breast carcinoma), Detroit 562 (pharyngeal carcinoma), T98G (astrocytoma), SKLMS-1 (leiomyosarcoma) and U-2-OS (osteosarcoma) cell lines harbour homozygous deletions of the p16 gene^{13,14}. The bacterially expressed p16 used as a control migrates more slowly because of a hexahistidine tag at the amino terminus. *b*, Immunofluorescence analysis of exponentially growing primary human fibroblasts, WI38, with the DCS-50 antibody shows nuclear and less intense cytoplasmic staining. *c*, Strong nuclear and cytoplasmic immunofluorescence revealed by staining RB-deficient J82 cells with DCS-50. Scale bar, 20 µm.

METHODS. Hybridoma clone DCS-50 producing an IgG1 antibody was isolated as described previously⁴ by immunizing BALB/c mice with bacterially produced histidine-tagged p16 purified by chromatography on chelating Sepharose¹⁹. Total protein extracts (50 µg) from the indicated cells were fractionated by SDS-PAGE in a 15% gel, transferred to nitrocellulose and probed with DCS-50 by enhanced chemiluminescence (ECL, Amersham) as detailed elsewhere⁴. The methods used for immunofluorescence were as previously described⁴.

§ To whom correspondence should be addressed.

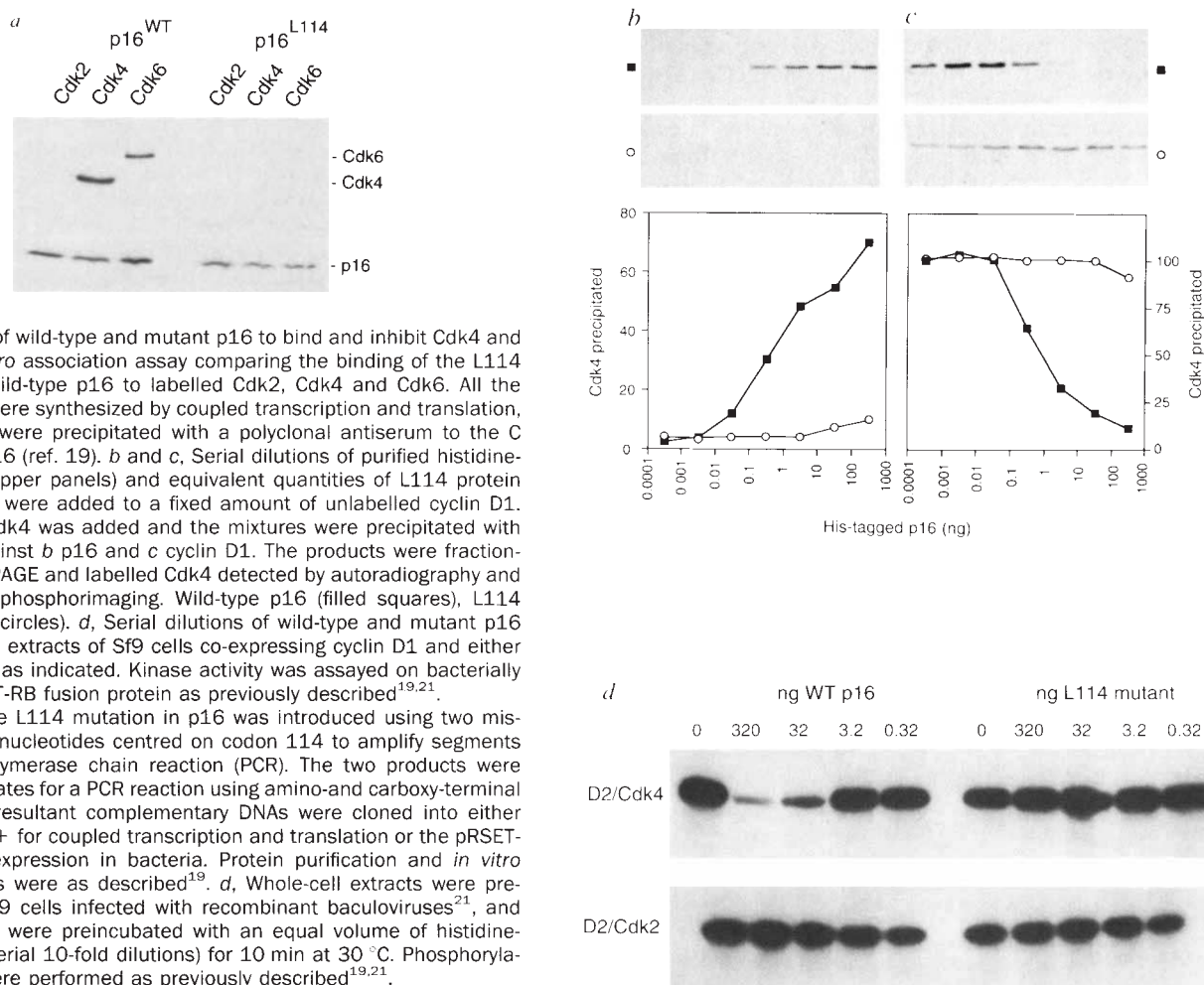


FIG. 2 Ability of wild-type and mutant p16 to bind and inhibit Cdk4 and Cdk6. **a**, *In vitro* association assay comparing the binding of the L114 mutant and wild-type p16 to labelled Cdk2, Cdk4 and Cdk6. All the components were synthesized by coupled transcription and translation, and mixtures were precipitated with a polyclonal antiserum to the C terminus of p16 (ref. 19). **b** and **c**, Serial dilutions of purified histidine-tagged p16 (upper panels) and equivalent quantities of purified histidine-tagged L114 protein (lower panels) were added to a fixed amount of unlabelled cyclin D1. ³⁵S-labelled Cdk4 was added and the mixtures were precipitated with antiserum against **b** p16 and **c** cyclin D1. The products were fractionated by SDS-PAGE and labelled Cdk4 detected by autoradiography and quantified by phosphorimaging. Wild-type p16 (filled squares), L114 mutant (open circles). **d**, Serial dilutions of wild-type and mutant p16 were added to extracts of Sf9 cells co-expressing cyclin D1 and either Cdk4 or Cdk2 as indicated. Kinase activity was assayed on bacterially expressed GST-RB fusion protein as previously described^{19,21}. **METHODS.** The L114 mutation in p16 was introduced using two mismatched oligonucleotides centred on codon 114 to amplify segments of p16 by polymerase chain reaction (PCR). The two products were used as templates for a PCR reaction using amino- and carboxy-terminal primers. The resultant complementary DNAs were cloned into either Bluescript KS+ for coupled transcription and translation or the pRSET-A vector for expression in bacteria. Protein purification and *in vitro* binding assays were as described¹⁹. **d**, Whole-cell extracts were prepared from Sf9 cells infected with recombinant baculoviruses²¹, and samples (5 µl) were preincubated with an equal volume of histidine-tagged p16 (serial 10-fold dilutions) for 10 min at 30 °C. Phosphorylation assays were performed as previously described^{19,21}.

CDKN2 in tumour cells suggests that p16 acts as a tumour suppressor. We show that wild-type p16 arrests normal diploid cells in late G1, whereas a tumour-associated mutant of p16 does not. Significantly, the ability of p16 to induce cell-cycle arrest is lost in cells lacking functional RB, including primary fibroblasts from *Rb*^{-/-} mouse embryos. Thus, loss of p16, overexpression of D-cyclins and loss of RB have similar effects on G1 progression, and may represent a common pathway to tumorigenesis.

To investigate functional links between p16, cyclin D1 and RB, the expression of endogenous p16 and the effects of exogenous p16 were analysed in a variety of normal and tumour-derived cells that differed in the status of the *RB1* gene. Immunoblotting with a monoclonal antibody against bacterially expressed p16 showed relatively low but detectable levels of the protein in normal diploid cells, significantly higher levels in RB-deficient tumour cell lines, and no detectable signal in several cancer cell lines harbouring wild-type RB (Fig. 1a). Immunofluorescence analyses revealed p16 in both the nucleus and cytoplasm¹⁵, with a much stronger signal in RB-deficient tumour cells than normal cells (Fig. 1b, c). In the human cell lines tested here, the absence of a p16 signal reflects homozygous deletion of the gene. The data are consistent with the inverse correlation between expression and/or mutation of RB and p16 in human tumour cells^{12,15,19}.

As a control for p16 function in cell cycle experiments, we constructed several mutant forms of p16 that reflect published sequence variants in human tumours. One such mutation, a C-to-T transition that occurs in three independent melanoma lines^{13,20}, changes proline 114 to leucine. In an *in vitro* binding assay¹⁹, the L114 mutant appeared unable to interact with either Cdk4 or Cdk6, whereas the wild-type p16 bound efficiently to

both Cdk4 and Cdk6, but not to Cdk2 (Fig. 2a). When bacterially expressed p16 was added to mixtures of cyclin D1 and Cdk4, the amount of Cdk4 co-precipitating with wild-type p16 increased in a dose-dependent manner, whereas negligible amounts of Cdk4 were co-precipitated with L114 (Fig. 2b). Conversely, when the mixtures were precipitated with a cyclin D1 antibody, wild-type p16 competed with cyclin D1 for binding to Cdk4 whereas L114 did not (Fig. 2c). Quantitation of the data by phosphorimaging indicated that the L114 mutant was 1,000-fold less effective in binding to Cdk4 (Fig. 2b, c).

To compare their properties as kinase inhibitors, dilutions of the two purified proteins were added to extracts of Sf9 insect cells co-expressing cyclin D1 and Cdk4 from baculovirus vectors^{19,21}. Under these conditions L114 did not inhibit the cyclin D2/Cdk4 kinase activity, whereas wild-type p16 was completely inhibitory, reducing the phosphorylation of the glutathione *S*-transferase (GST)-RB substrate to that seen in control reactions (Fig. 2d). Neither protein had any effect on the kinase activity of cyclin D2/Cdk2 (Fig. 2d).

We next asked whether wild-type and mutant p16 differed in their ability to cause cell cycle arrest when microinjected into viable cells. Synchronized fibroblasts and myoepithelial or luminal epithelial cells from normal breast were microinjected in early G1 with wild-type and mutant p16, prepared as soluble GST-fusion proteins. Cells injected with wild-type p16 were generally prevented from entering S-phase, as judged by bromodeoxyuridine incorporation, in contrast to the neighbouring non-injected cells (Fig. 3a, b). The L114 mutant had no effect on the onset of DNA synthesis in any of the three diploid cell types (Fig. 3b).

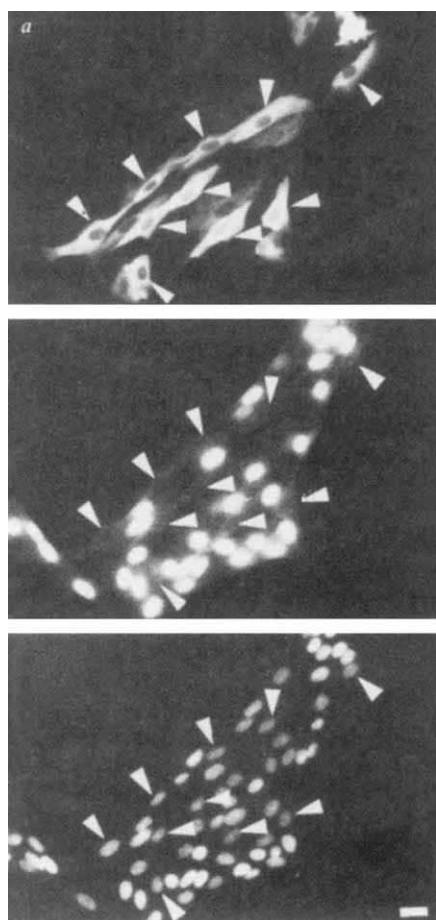
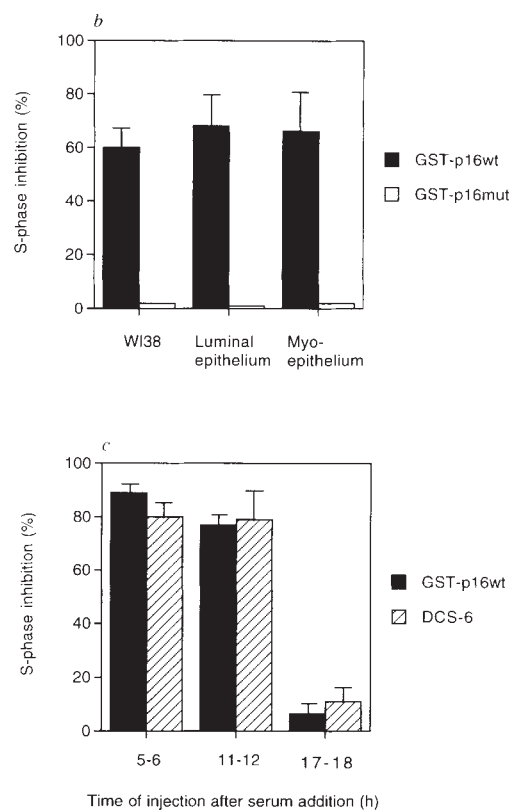


FIG. 3 Microinjection of wild-type and mutant p16 into human diploid cells. *a*, Immunofluorescence analysis of luminal breast epithelium injected with wild-type GST-p16 and marker immunoglobulin. Top, arrowheads indicate immunoglobulin-positive, microinjected cells. Middle, identical field stained for bromodeoxyuridine. Most microinjected cells (arrowheads) are prevented from S-phase entry, whereas most non-injected cells incorporated bromodeoxyuridine. Bottom, nuclear counterstaining with Hoechst. *b*, Graphical representation of cell-cycle inhibition by wild-type p16 but not L114 in WI38 fibroblasts and breast luminal or myoepithelial cells. *c*, WI38 cells injected with either p16 or with cyclin D1 antibodies show inhibition of S-phase entry when injected in early G1 (5–6 h), mid-to-late G1 (11–12 h) but not at the G1/S transition (17–18 h).

To determine when in the cell cycle the influence of p16 is manifest, quiescent WI38 fibroblasts were microinjected at different times after serum addition. Introducing GST-p16 at 5–6 h or 11–12 h after serum stimulation severely inhibited S-phase entry but had little effect after 17–18 h when most cells were approaching the G1/S transition (Fig. 3*c*). The effects observed with p16 were similar to the S-phase inhibition by antibody-mediated neutralization of cyclin D1, performed in parallel (Fig. 3*c*; and refs 1, 4). In cells released from an aphidicolin block, microinjection of p16 in S-phase did not prevent progression through G2/M (not shown). Thus p16 can only cause cell cycle arrest up to a certain stage in late G1, presumably corresponding to a checkpoint executed by cyclin D/Cdk complexes.

Because RB is a substrate for these kinases, we measured the effects of ectopic p16 expression in sarcoma cell lines that have different patterns of RB expression²². Plasmids encoding wild-type and mutant p16 were introduced by transfection and after 48 h the proportions of cells in G1, S and G2/M were evaluated by flow cytometry. Wild-type p16 caused U-2-OS (RB⁺/p53⁺) and SKLMS-1 (RB⁺/p53⁺) cells to accumulate in G1 but had no significant effect on Saos-2 (RB[−]/p53[−]) cells (Fig. 4*a*). In contrast, the results with L114 were indistinguishable from the



METHODS. The cDNAs for wild-type p16 and L114 were subcloned into pGEX-2TK (Pharmacia), expressed in *Escherichia coli* BL21 (DE3) pLysS, and the respective fusion proteins purified by affinity chromatography. Luminal and myoepithelial cells were isolated from reduction mammaplasty tissue and cultured in a defined serum-free medium as described²⁴. Early passage cells were synchronized by growth factor deprivation and restimulation in complete medium. Cells were co-injected with GST-p16 and nonspecific mouse immunoglobulin (each at 1 mg ml^{−1}) and S-phase entry assayed by bromodeoxyuridine incorporation as previously described²². Percentage of S-phase inhibition was calculated from a minimum of 100 injected cells according to the formula: S-phase inhibition = (A-B)/A × 100, where A is the percentage of surrounding non-injected cells incorporating bromodeoxyuridine and B is the percentage of microinjected cells incorporating bromodeoxyuridine. The combined results of three independent experiments are shown. Microinjection of the DCS-6 anti-cyclin D1 antibody was as previously described⁴.

vector control (Fig. 4*a*). The growth-inhibitory effect of p16 in U-2-OS cells was reversed by co-transfection of adenovirus E1A but not by a mutant form of E1A that is unable to bind RB (not shown). These results complement recent reports that p16 blocks transformation by Ras+Myc but not by Ras+E1A²³, and inhibits colony formation of RB-positive but not RB-negative cells^{9,15}. We also performed microinjection experiments in primary fibroblast cultures established from *Rb*^{−/−} mouse embryos and their *Rb*^{+/+} littermates. Microinjection of wild-type p16 caused a significant degree of G1 arrest in RB-positive mouse cells but had little effect in early- or late-passage fibroblasts from *Rb*^{−/−} embryos (Fig. 4*b*). The L114 mutant was unable to cause G1 arrest in any of the mouse cells tested.

Taken together, our data show that p16 is important in negative regulation of the mammalian cell cycle in late G1, grossly coinciding with cyclin D/Cdk function and the phosphorylation of RB. Missense mutations in p16 that impair its interaction with Cdk4 and Cdk6 may provide a growth advantage to a tumour cell by removing the constraints on cyclin D/Cdk activity, thereby promoting the inactivation of RB. Because RB lies downstream of p16 and cyclin D, abrogation of either p16 or cyclin D1 activity has no effect in cells that are already deficient

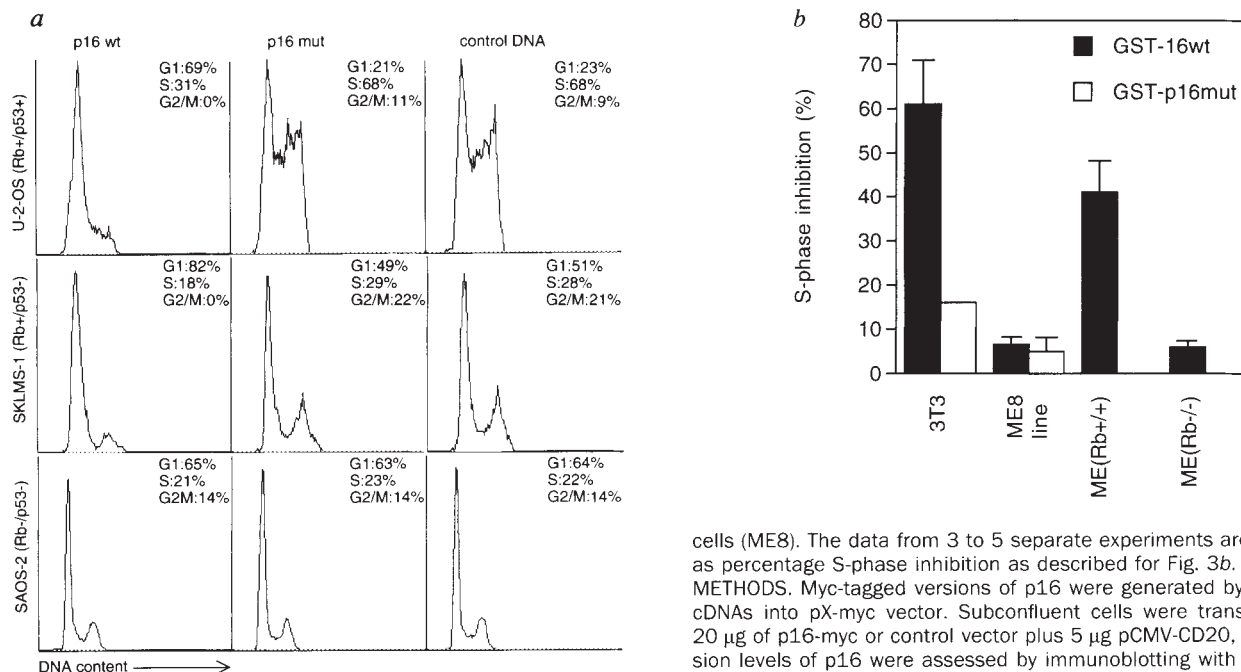


FIG. 4 G1 inhibitory effect of p16 is restricted to cells expressing functional RB. **a**, The indicated human cell lines were transfected with plasmids encoding wild-type p16, the L114 mutant, or control vector, together with a CD20 expression plasmid. Cells were collected after 48 h, stained for CD20 antigen and DNA content, and analysed by multiparameter flow cytometry. The data are presented as histograms of 5,000 CD20-positive cells in which cell number is plotted against DNA content. **b**, Effects of microinjecting GST-p16 into mouse 3T3 cells, early-passage $Rb^{+/+}$ or $Rb^{-/-}$ embryo fibroblasts, or late-passage $Rb^{-/-}$

cells (ME8). The data from 3 to 5 separate experiments are expressed as percentage S-phase inhibition as described for Fig. 3b.

METHODS. Myc-tagged versions of p16 were generated by subcloning cDNAs into pX-myc vector. Subconfluent cells were transfected with 20 μ g of p16-myc or control vector plus 5 μ g pCMV-CD20, and expression levels of p16 were assessed by immunoblotting with monoclonal antibody 9E10. Analyses of cell cycle effects were performed as described previously²⁵, and the average of 3 experiments is shown. **b**, Homozygous $Rb^{+/+}$ and $Rb^{-/-}$ littermate mouse embryos were obtained as described²⁶, and fibroblast cultures were prepared from 13-day embryos. Cells were synchronized by serum removal and addition. Mouse embryo cells at passages 6–10 were microinjected immediately after serum addition to avoid the possibility of passing through a p16-controlled checkpoint that might occur earlier in $Rb^{-/-}$ cells. In the case of NIH3T3 and late-passage ME8 cells, microinjection was performed in early G1. The effects were evaluated 20 h later, as for Fig. 3b.

in RB (Fig. 4 and ref. 22). These results are consistent with the emerging role of p16 as a tumour suppressor, and the assays used here may help resolve the issue of cancer-promoting mutations versus silent polymorphisms of the p16 gene. □

Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition

James Koh*, Greg H. Enders*†, Brian David Dynlacht* & Ed Harlow*

* Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, Massachusetts 02129, USA
† Gastrointestinal Unit, Massachusetts General Hospital, Fruit Street, Boston, Massachusetts 02114, USA

THE cyclin-dependent kinase inhibitor p16 is a candidate tumour-suppressor protein that maps to a genomic locus strongly associated with familial melanoma and other tumour types^{1–3}. Screening of primary tumours and linkage analysis of familial melanoma pedigrees have identified many potential mutations in *p16*, but the functional significance of these sequence variants has remained unclear^{1,3–9}. We report here that p16 can act as a potent and specific inhibitor of progression through the G1 phase of the cell cycle, and we demonstrate that several tumour-derived alleles of *p16* encode functionally compromised proteins. The ability of p16 to arrest cell-cycle progression generally correlates with inhibition of cyclin D1/Cdk4 kinase activity *in vitro*, with two exceptions among the alleles tested. *In vivo*, the presence of functional retinoblastoma protein appears to be necessary but may not be sufficient to confer full sensitivity to p16-mediated growth arrest. Our results provide support for the notion that p16 is an important cell-cycle regulator whose inactivation contributes to the outgrowth of human tumours.

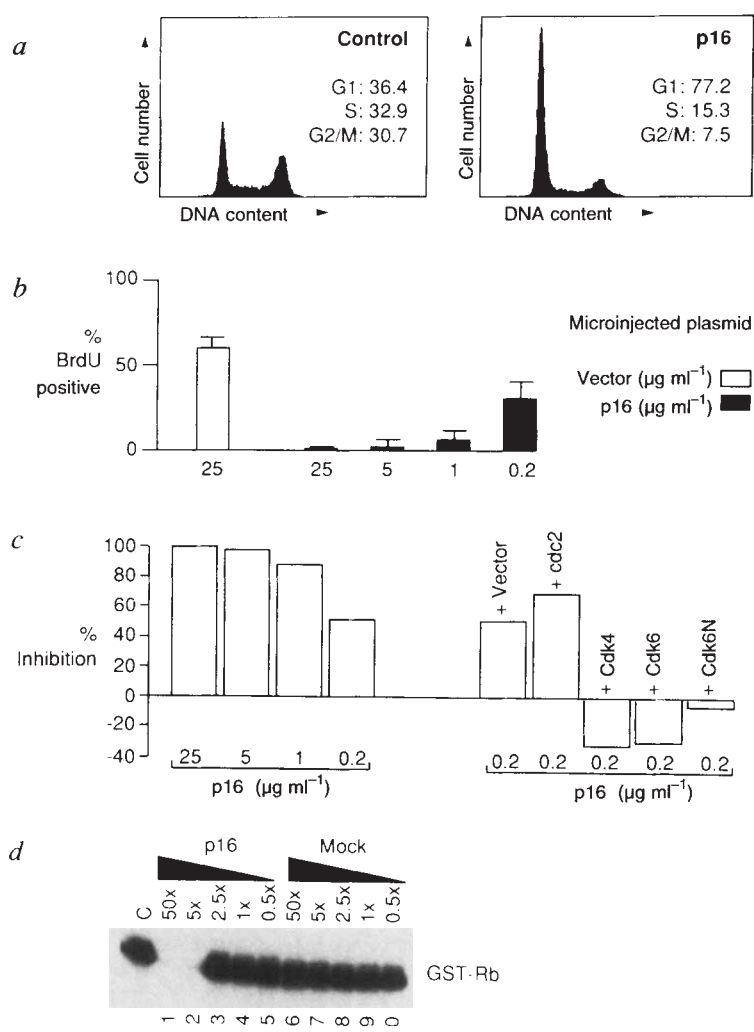
Received 18 January; accepted 23 March 1995.

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ACKNOWLEDGEMENTS. We thank C. Sherr for providing baculovirus expressing cyclin D2 and Cdk4, M. Meyerson for the GST-RB construct, D. Morgan for the Cdk2 virus, E. Nigg for the pX-myc vector, G. Evan for the 9E10 anti-Myc antibody, K. Helin for the CD20 plasmid, O. Petersen for diploid breast epithelial cells, and H. te Riele and A. Berns for the heterozygous $Rb^{+/+}$ male mice. We also thank C. Dickson, G. Evan and A. Sinclair for comments on the manuscript.

FIG. 1 Functional assays for p16 activity. **a**, Flow cytometry analysis of transfected U2OS cells. Asynchronous populations of U2OS cells in log-phase growth were transiently transfected with a pCMV expression plasmid containing no insert (control; left) or containing the full-length human p16 cDNA (right). A CD20 expression construct in the same plasmid vector was co-transfected at a molar ratio of 1:8 relative to the test constructs. Each transfected population is depicted in histogram form, with cell number plotted on the y-axis and relative DNA content on the x-axis. The control and p16 histograms contain data from 4,578 and 6,453 cells, respectively. **b**, Inhibition of DNA synthesis in NIH3T3 cells by microinjection of a plasmid expressing p16. Quiescent cells (passage 10–20; gift from C. Sherr) were restimulated and co-injected 1–5 h later with a pCMV plasmid encoding β -galactosidase (β -gal) at $25 \mu\text{g ml}^{-1}$ and the indicated plasmids at the concentrations shown. DNA synthesis was monitored by BrdU incorporation. The percentage of β -gal-positive cells that were BrdU-positive at 24 h was determined using immunofluorescent staining. Depicted are the average values and standard deviations derived from at least three independent experiments. A total of 648 β -gal positive cells were counted. Under these conditions, 80 to 90% of uninjected cells are BrdU-positive. **c**, 'Rescue' of p16 inhibition by co-injection of plasmids expressing CDKs. Right, NIH3T3 cells were microinjected with the designated plasmids in the presence or absence of $0.2 \mu\text{g ml}^{-1}$ plasmid encoding p16. The percentage of expressing cells (β -gal positive) that were BrdU-positive was determined. The value obtained in the presence of p16 was subtracted from that obtained in its absence and divided by the latter to yield the per cent inhibition by p16 observed in the presence of each co-injected plasmid. The vector and Cdk6 plasmids were injected at $50 \mu\text{g ml}^{-1}$ and the Cdc2, Cdk4 and Cdk6N plasmids were injected at $25 \mu\text{g ml}^{-1}$. Each experiment was done at least twice and a representative result is shown. Data from a total of 410 β -gal-positive cells is presented. Left, per cent inhibition seen with injection of p16 alone, derived from the data in **b**, are presented for comparison. **d**, Kinase inhibition assay using recombinant components. Purified baculovirus-expressed cyclin D1/glutathione-S-transferase (GST)-Cdk4 complexes were combined with varying amounts of purified bacterially expressed His-tagged p16 protein, and kinase activity was assayed using GST-Rb large pocket as a substrate. The molar ratio of p16 protein relative to the amount of cyclin/CDK complex in each reaction is indicated above the lanes. In the mock lanes, column eluates from empty vector-transformed bacteria were used at dilutions equivalent to the p16 fractions as a control for co-purifying bacterial components. The lane labelled 'C' is a control reaction containing no inhibitor protein.

METHODS. The human p16 cDNA was amplified by reverse transcriptase PCR from WI38 RNA and subcloned into the *Bam*HI site of the mammalian expression vector pCMV-Neo²⁴. The coding sequence of the cDNA corresponds to GenBank entry L27211 (ref. 10). U2OS cells were transfected and analysed as described¹¹, except that the cells were harvested 24 h after removal of the DNA precipitates. In all FACS results reported here, data were generated from a minimum of 2,000 CD20⁺ cells per sample. Microinjection was performed as described²⁵. Hi5 cells (Invitrogen, San Diego CA) were harvested 40 h after infection with baculoviruses encoding cyclin D1 (gift from D. Beach) and a GST-



Cdk4 fusion protein (gift from W. Harper). Cell pellets were resuspended in D buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glycerol) containing 0.2 mM AEBSF, 1 mM DTT, $5 \mu\text{g ml}^{-1}$ leupeptin, and $5 \mu\text{g ml}^{-1}$ aprotinin and disrupted by sonication. Lysates were cleared by centrifugation and supernatants incubated for 30 min with glutathione-Sepharose beads at 4°C , pellets were washed four times with D buffer, and protein complexes were eluted with 20 mM reduced glutathione in 100 mM Tris-HCl, pH 7.9, 120 mM NaCl. Eluates were then dialysed against D buffer containing 1 mM DTT and 0.2 mM AEBSF. N-terminal His-tagged p16 protein was prepared from bacteria transformed with a pQE9 (Qiagen) construct expressing the full-length human cDNA. Extracts were prepared under denaturing conditions and purified protein was recovered using a commercially prepared Ni-NTA resin (Qiagen). Kinase was assayed as described²⁶.

To investigate the role of p16 in regulating cell-cycle progression, we transfected a full-length human p16 cDNA¹⁰ expression construct into the human osteosarcoma cell line U2OS. Forty-eight hours after transfection, DNA content was assayed by flow cytometry to determine cell cycle distribution, with gating to restrict analysis to cells expressing a co-transfected marker (CD20; ref. 11). As shown in Fig. 1a, transfection with p16 caused a marked accumulation of cells in G1, consistent with an arrest at or before the G1/S boundary. This result suggested that overexpression of p16 is sufficient to arrest the cell cycle. To test the effect of p16 expression in a synchronized, untransfected cell population, we used a plasmid microinjection assay in NIH3T3 cells. Cells arrested by serum starvation were micro-

injected shortly after synchronous release, and entry into S phase was monitored by bromodeoxyuridine (BrdU) uptake in cells positive for a co-injected marker. Microinjection of the p16 expression construct blocked progression into S phase in a dose-dependent manner (Fig. 1b). Using prior estimates of the amount of DNA delivered in this setting¹², 50% inhibition of DNA synthesis could be achieved with injection of ~ 1 –4 copies of the p16 plasmid per cell. Microinjection of the p16 construct into S phase cells did not influence progression through the G2/M boundary (data not shown).

The specificity of the p16 effect was tested by co-injection of several cdk constructs to assay for phenotypic rescue of the arrested cells (Fig. 1c). Because p16 interacts only with Cdk4

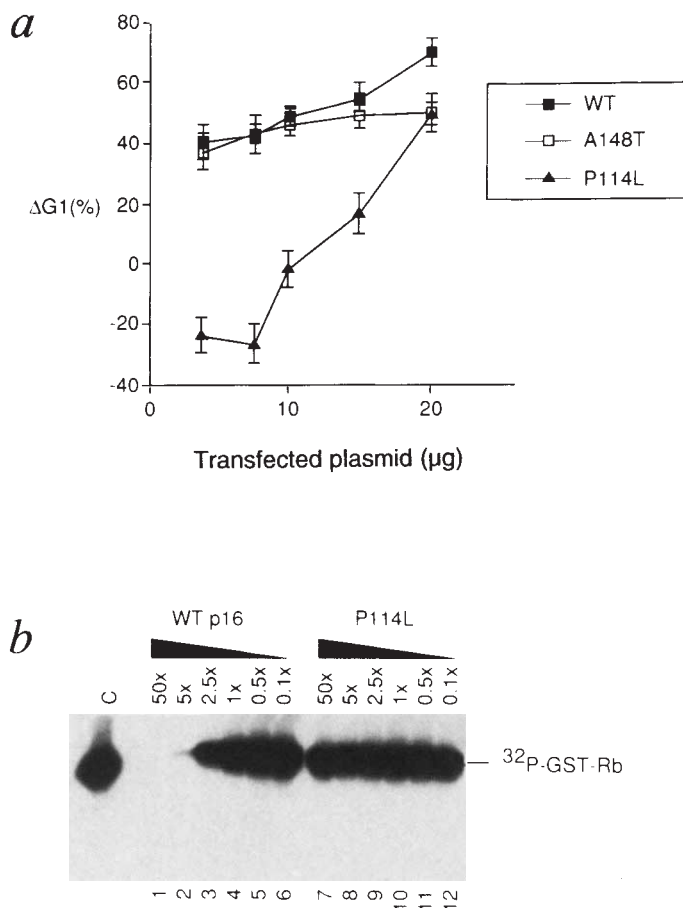


FIG. 2 A tumour-derived *p16* allele encodes a functionally defective protein. **a**, U2OS transfection assay comparing growth-arrest properties of wild-type *p16* to two sequence variant alleles. The nomenclature indicates the location and identity of amino-acid substitutions (see text). FACS data from U2OS cells transfected with decreasing amounts of the indicated *p16* constructs are plotted as a function of $\Delta G1$, the per cent change in the proportion of cells in G1 relative to the vector control sample. $\Delta G1$ is defined as [(proportion of cells in G1, test sample) – (proportion of cells in G1, control)]/(proportion of cells in G1, control) $\times 100$. Each data point represents the mean $\pm$ s.d. from at least three independent experiments. **b**, Kinase inhibition assay using recombinant components. Purified cyclin D1/GST-Cdk4 complexes were combined with varying amounts of purified bacterially expressed wild type or P114L mutant His-tagged *p16* protein, and kinase activity was assayed with GST-RB large pocket as a substrate. The molar ratio of *p16* protein relative to the amount of cyclin/CDK complex in each reaction is shown above each lane. The control lane, labelled C, contains no inhibitor protein.

METHODS. U2OS cells were transfected, processed and analysed as for Fig. 1. In experiments in which less than 20 μ g of a test construct was used, the total amount of DNA in the transfection is maintained at 24 μ g by addition of vector plasmid. Kinase assays were as for Fig. 1.

and Cdk6 among the known CDKs^{10,13}, co-injection of plasmids encoding Cdk4 and Cdk6 should prevent *p16*-mediated growth arrest, whereas plasmids encoding non-cognate CDKs such as Cdc2 should have no effect. Under injection conditions that produce limiting levels of *p16*, co-injection of either *cdk4* or *cdk6*, but not *cdc2*, expression plasmids prevented *p16*-mediated growth arrest. Rescue probably occurs by binding and titration of the *p16* protein rather than by complementation of kinase activity, because a construct encoding a Cdk6 variant bearing a mutation predicted to disrupt its catalytic centre (Cdk6N) retained the ability to prevent growth arrest¹¹. These results verify the specificity of the *p16* arrest and indicate that growth inhibition by *p16* requires an accessible CDK-interacting domain.

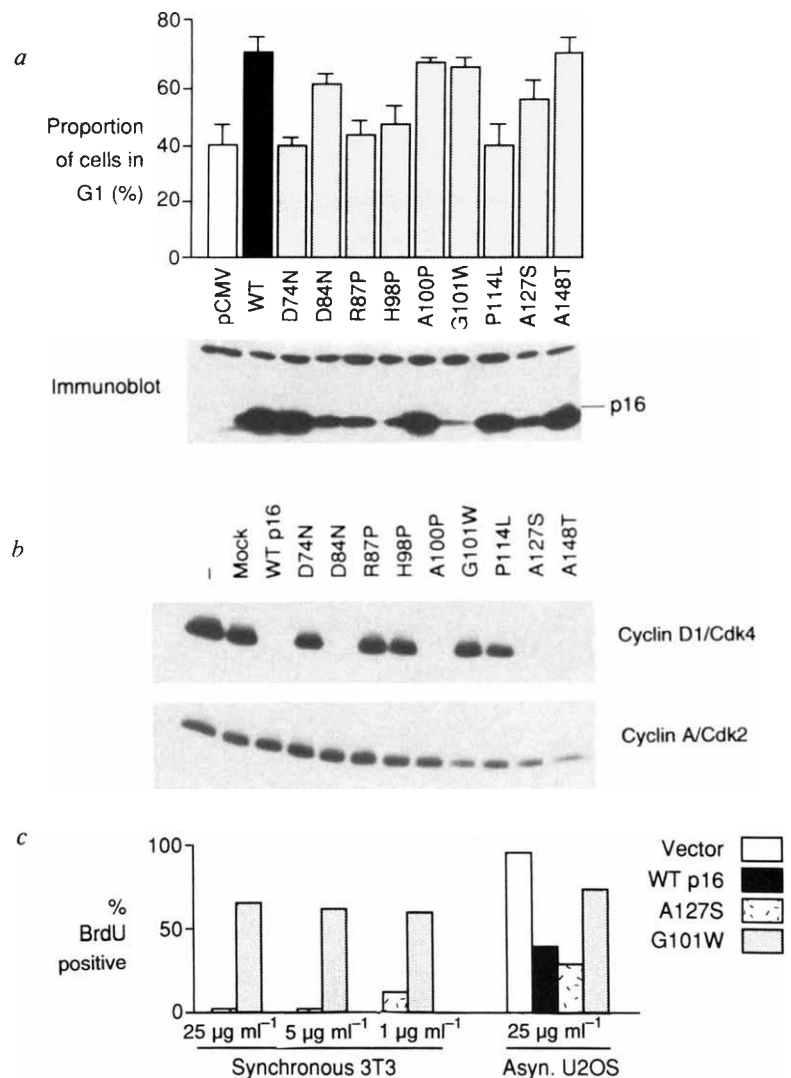
To demonstrate the kinase-inhibitory activity of *p16* in a defined biochemical system, we purified bacterially expressed *p16* protein. Recombinant *p16* protein inhibited the ability of purified cyclin D1/GST-Cdk4 complexes to phosphorylate the carboxy-terminal fragment of the retinoblastoma protein (Fig. 1d). The degree of kinase inhibition does not appear to be linear with respect to molar ratio of *p16* (compare lanes 2 and 3), perhaps indicating a stoichiometric threshold mode of action, as has been suggested for p21 (ref. 14).

Having established several measurable criteria for wild-type *p16* activity, we tested whether our assays could distinguish functionally compromised mutant alleles from sequence polymorphisms. We generated two missense substitution alleles of *p16* identified previously¹. The first allele, a proline-to-leucine substitution at codon 114 (P114L), is present in a hemizygous state in one melanoma cell line and is found opposite a termination mutant allele in another melanoma cell line, both contexts suggesting that P114L may be functionally disabled. The second allele, A148T, has since been shown to be a common sequence polymorphism that does not co-segregate with disease in melanoma kindreds⁶. Titrations of the wild-type (WT), A148T, and P114L constructs or proteins were tested in all three of the assays already described (Fig. 2 and data not shown). Transfection with 3.8 μ g wild-type *p16* construct was sufficient to induce a substantial accumulation of cells in G1 (Fig. 2a). The A148T allele behaved similarly to the wild-type construct, consistent with its designation as a polymorphic sequence variant. In contrast, the P114L construct promoted G1 arrest only at the highest levels of transfecting plasmid tested, rapidly losing effect as input levels were decreased. Microinjection of the P114L construct into NIH3T3 cells required a DNA concentration two logs higher than the wild-type construct to achieve 50% inhibition of BrdU incorporation (Fig. 1b and data not shown). The P114L protein is also defective in its ability to inhibit cyclin D1/Cdk4 kinase activity, producing no inhibition at a molar excess 10 times greater than that required for full inhibition by the wild-type protein (Fig. 2b: compare lanes 2 and 7). The growth arrest provoked by high concentration transfection of the P114L construct may be due to residual activity: at a high molar excess in the range of 2,500:1, P114L mutant protein can inhibit cyclin D1/Cdk4 but not cyclin A/Cdk2 kinase activity (data not shown). These results demonstrate the specificity of our assays and identify the P114L missense substitution as a functionally compromised mutant allele.

We extended our analysis of candidate *p16* mutant alleles to seven additional missense substitutions. D74N is a *de novo* acquired somatic mutation isolated independently from tumours of the oesophagus and bladder^{3,4}. D84N was found in a survey of oesophageal squamous cell carcinomas³. R87P and G101W have been strongly associated with familial melanoma in linkage studies⁶. H98P, A100P, A127S and A119S are sequence variants isolated from primary melanomas, but the frequency of these alleles in control populations has not been reported (A. Kamb, personal communication). The mutants were tested in growth arrest and kinase inhibition assays (Fig. 3a, b). As a control for specificity, the lower panel of Fig. 3b shows that none of the *p16* proteins affected the kinase activity of cyclin A/Cdk2 complexes. The tested alleles fall into three categories: alleles that were functionally indistinguishable from wild type and therefore probable polymorphisms (A100P, A148T), alleles that were functionally inactive in both tests and therefore are loss-of-function alleles (D74N, R87P, H98P, P114L), and three that gave intermediate or discordant results (D84N, G101W, A127S). The D84N and A127S proteins effectively inhibited cyclin D1/Cdk4 kinase activity but were intermediate in their ability to cause growth arrest. The A127S construct was as effective as wild type in causing growth arrest upon microinjection into either NIH3T3 or U2OS cells (Fig. 2c). Therefore, the intermediate growth arrest phenotype of A127S upon transfection was likely to be due to lower expression levels (compare to the level of wild-type protein

FIG. 3 Functional analysis of *p16* alleles. *a*, Growth arrest assay in U2OS cells. 10 μg of an expression construct encoding each of the indicated *p16* alleles was transfected into an asynchronous culture of U2OS cells and cell-cycle distribution was assessed by FACS as in Fig. 1. The proportion of cells in the G1 phase of the cell cycle is graphed on the y-axis. WT, wild-type *p16* construct. The lanes on the accompanying immunoblot are aligned with the graph to show *p16* expression levels after transfection with the respective constructs. The upper band present in all lanes is a cross-reacting species that serves as an internal control for equivalent loading. U2OS cells lack endogenous *p16* as determined by RT-PCR (data not shown). *b*, Kinase assays testing for inhibitory activity. His-tagged *p16* proteins encoded by each of the indicated alleles were assayed for the ability to inhibit the cyclin D1/Cdk4 and cyclin A/Cdk2 kinase activity, using GST-RB as a substrate for both enzymes. First lane, no inhibitor added; second lane, (mock) indicates the control vector-derived fraction described in Fig. 1d legend. *c*, Inhibition of DNA synthesis by microinjection of plasmids expressing *p16* mutants into synchronized NIH3T3 cells and asynchronous U2OS cells. Left, plasmids expressing *p16* mutants A127S and G101W were microinjected into serum-starved and restimulated NIH3T3 cells under the same conditions as in Fig. 1b. Right, asynchronous U2OS cells were injected with the indicated plasmids at 25 $\mu\text{g ml}^{-1}$. BrdU was added 24 h after injection, and the cells were fixed after another 24 h. Under these conditions, approximately 95% of uninjected U2OS cells were BrdU-positive. Data from a total of 161 β -gal-positive NIH3T3 and 335 U2OS cells are presented.

METHODS. Transfections and microinjection assays were done as described above. The immunoblot was prepared from total cell lysates of transfected U2OS cells as described²⁷. 25 μg total protein per lane was loaded and resolved on a 15% SDS-polyacrylamide gel, transferred to an Immobilon M membrane (Millipore) and probed with a commercially acquired rabbit polyclonal anti-*p16* antibody (Pharmingen).



in the immunoblot in Fig. 3a), and this allele should be classified as a polymorphism. D84N reproducibly caused partial arrest when microinjected into NIH3T3 cells (data not shown), indicating that this mutant has an *in vivo* defect not apparent under the *in vitro* kinase assay conditions.

The G101W mutant represents a converse exception to the correlation between cyclin D1/Cdk4 kinase inhibitory activity and the ability to provoke growth arrest. Protein produced by this allele does not inhibit cyclin D1/Cdk4 kinase activity *in vitro* but is nonetheless competent to arrest U2OS cells in G1. By microinjection, however, the G101W construct fails to block S-phase entry in NIH3T3 cells and shows only modest inhibition in U2OS cells (Fig. 3c). As was the case for the P114L protein, the G101W protein retains residual activity, specifically inhibiting cyclin D1/Cdk4 only when present at substantial molar excess (data not shown). Furthermore, titration experiments reveal a sharp drop-off in growth arrest when less transfecting plasmid is used (data not shown). Taking into consideration the kinase data, the microinjection results and the strong genetic evidence for an association between the G101W mutation and familial melanoma⁶, we conclude that this allele encodes a functionally compromised mutant whose defects can be masked by overexpression. It is also possible that in some cellular environments *p16* may be able to exert growth regulatory effects independent of cyclin D1/Cdk4 kinase inhibition. The behaviour of mutant alleles such as G101W and D84N underscores the need for multiple independent and titratable assays when testing for *p16* function.

We next tested a panel of cell lines to determine whether sensitivity to *p16*-mediated growth arrest was a uniform characteristic among cultured cells (Fig. 4a). The cell lines U2OS, T98G, ML-1, and U118 could be arrested by *p16* overexpression, but SAOS-2 and C33a cells showed no change in cell-cycle distribution upon transfection with *p16*. Although there are many potential explanations for this differential sensitivity, one correlating factor is the presence of a functional allele of the retinoblastoma gene *RB-1*. Given that there is substantial circumstantial evidence linking the action of cyclin D/Cdk4 and cyclin D/Cdk6 kinases to the inactivating phosphorylation of the retinoblastoma protein, RB (refs 10, 15–19), *p16* may require RB as a downstream effector in mediating growth arrest. A survey of lung-cancer cell lines has shown a striking inverse correlation between the presence of *p16* and wild-type RB, suggesting that deletion of both regulators may be functionally redundant²⁰. To test whether RB is required for sensitivity to *p16*, we assayed for growth arrest upon microinjection into matched sets of primary mouse embryonic fibroblasts (MEFs) derived from wild-type or *Rb*^{-/-} nullizygous mice²¹. As shown in Fig. 4b, microinjection of a *p16* expression construct into synchronized *Rb*^{-/-} nullizygous MEFs did not affect progression to S phase. In contrast, *Rb* wild-type MEFs are inhibited by *p16*, although the arrest is partial at the population level. BrdU incorporation levels are generally lower and more variable in wild-type MEFs than in their *Rb*^{-/-} nullizygous counterparts following synchronization by serum deprivation, but in three independent experiments the relative inhibition of BrdU incorporation mediated by *p16* in

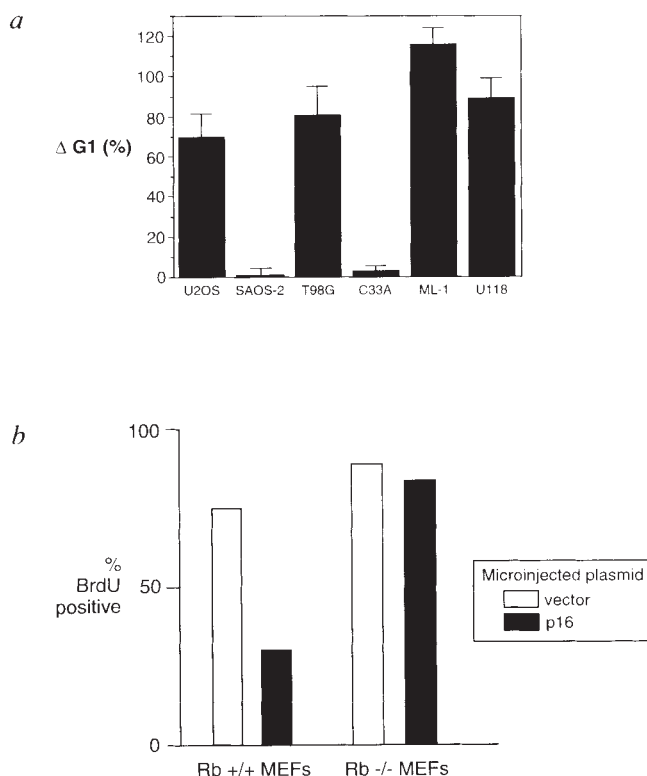


FIG. 4 Sensitivity to p16-mediated cell-cycle arrest correlates with RB status. *a*, Cultured tumour cell lines differ in their sensitivity to p16. 20 μ g wild-type p16 expression plasmid was transfected into the indicated cell lines and cell-cycle distribution was subsequently determined by FACS. Δ G1 was calculated as for Fig. 2a. Values shown are the mean $\pm$ s.d. of at least three independent experiments for each cell line. The cell lines U2OS, T98G, ML-1 and U118 contain endogenous functional RB protein; the cell lines SAOS-2 and C33a lack functional RB protein. Both p16 and p15 transcripts could be detected by RT-PCR from SAOS-2 and C33a RNA, but neither transcript was detectable in U2OS, T98G, ML-1 or U118 RNA (data not shown). *b*, Inhibition of DNA synthesis in wild-type and Rb nullizygous mouse embryo fibroblasts by microinjection of a p16 expression plasmid. Fibroblasts collected from wild-type and Rb nullizygous mouse embryo littermates were arrested by serum deprivation. Cells were restimulated and microinjected with the p16 expression plasmid or the empty vector at 25 μ g ml⁻¹, and entry into S phase was monitored by BrdU incorporation.

METHODS. Fibroblasts were collected from 13-day-old mouse embryos by standard techniques and used during passages 4–7. Rb genotype was determined as described²¹. Serum arrest, microinjection and processing are described in Fig. 1b, except that cells were fixed 26–31 h after restimulation. About 80–90% of uninjected cells incorporated BrdU; this figure presents data from 103 β -gal-positive cells; results from a total of 1,289 β -gal positive MEFs are described in the text.

wild-type MEFs was significantly different both from the complete inhibition observed in NIH3T3 cells under the same injection conditions (Fig. 1b), and from the lack of inhibition seen in Rb^{-/-} nullizygous MEFs (Fig. 4b and data not shown). The intermediate sensitivity to p16 inhibition in wild-type MEFs cannot be accounted for by inadequate expression of p16 or Rb, because high levels of p16 protein could be demonstrated by immunofluorescent staining of the injected cells and co-expression of Rb failed to confer full inhibition (data not shown). These results are consistent with current models positioning Rb as a downstream effector of p16, but the incomplete inhibition observed in Rb wild-type MEFs suggests that Rb may not be the only relevant variable in determining sensitivity to p16-mediated arrest.

In summary, we have shown that p16 can act as an inhibitor of progression through the G1 phase of the cell cycle, consistent

with reports describing a role for p16 in the regulation of cellular proliferation^{22,23}. We have used both biological and biochemical assays to demonstrate functional defects in several disease-associated alleles of p16, including a tumour-derived *de novo* somatic mutant. These data support a direct connection between inactivation of p16 and tumour susceptibility, confirming the status of p16 as a tumour suppressor. □

Received 18 January; accepted 11 May 1995.

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ACKNOWLEDGEMENTS. We thank D. Beach and W. Harper for gifts of cyclin D1 and GST-Cdk4 baculovirus constructs, respectively; T. Jacks for Rb nullizygous mice; M. Meyerson and R. Hurford for help in preparing murine embryonic fibroblasts; and D. Dombkowski for technical assistance with flow cytometry. This work was supported by grants from the NIH. J.K. is a Leukemia Society of America Fellow, G.H.E. is the recipient of a Clinical Investigator Award from the National Cancer Institute, B.D.D. is a Damon Runyan–Walter Winchell Cancer Fund Fellow, and E.H. is an American Cancer Society Research Professor.

A two-step mechanism for 5' and 3' splice-site pairing

Maria Dolores Chiara & Robin Reed

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

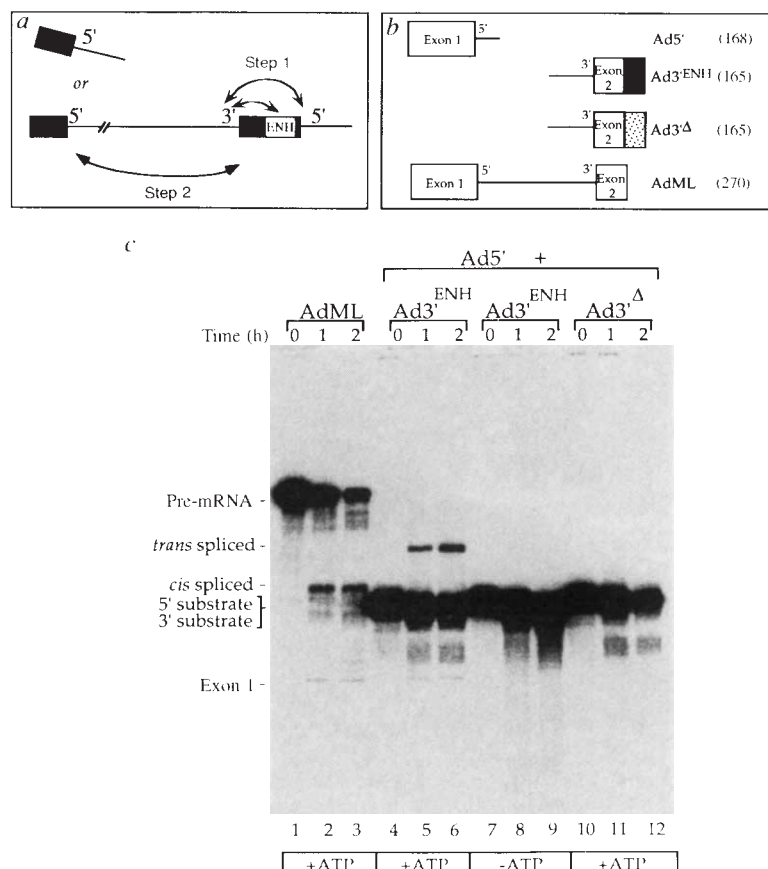
A FUNDAMENTAL question in the splicing of precursor messenger RNA is how the 5' and 3' splice sites are recognized and paired during the splicing reaction. It has been proposed that spliceosome assembly in metazoan pre-mRNAs can be initiated through interaction between the 3' splice site and specific sequence elements on the downstream exon (an exonic enhancer or a 5' splice site)¹. Pairing of the intronic 5' and 3' splice sites occurs subsequently. We report here that 5' and 3' splice sites located on separate synthetic pre-mRNA substrates can be efficiently *trans*-spliced if the 3' *trans*-splicing substrate contains these downstream sequence elements. Moreover, selection of the *trans* 5' splice site can occur after the second pre-spliceosomal complex A has assembled on the 3' *trans*-splicing substrate. Thus our data demonstrate that 5' and 3' splice-site pairing in metazoans can occur in two distinct steps.

To determine whether a *trans* splicing reaction can occur if the 3' substrate contains an exonic enhancer^{2,3} or a downstream 5' splice site¹ (Fig. 1a), we constructed synthetic 5' and 3' *trans* splicing substrates (Fig. 1b). Figure 1c shows that *trans* splicing does indeed occur efficiently between the 5' substrate (Ad5') and a 3' substrate containing an enhancer (Ad3^{ENH}) (lanes 4 to 6),

FIG. 1 An exonic enhancer promotes *trans* splicing *in vitro*.

a, Two-step model for 5' and 3' splice-site pairing¹. In step 1, the 3' splice site interacts with the downstream 5' splice site and/or exonic enhancer (ENH). In step 2, the upstream 5' splice site is selected. **b**, Structures of the AdML substrates. The sizes in nucleotides of the RNAs are indicated in brackets. The 5' substrate (Ad5') contains exon 1 (129 nucleotides) and the 5' portion of the intron (39 nucleotides). The 3' substrates contain the branchpoint sequence and 3' splice site (80 nucleotides) and exon 2 (45 nucleotides). Ad3^{ENH} also contains the avian sarcoma leukosis virus enhancer (40 nucleotides)¹⁷ inserted at the 3' end of exon 2, and Ad3^Δ RNA contains a sequence of the same size that lacks enhancer activity¹⁷. **c**, Splicing time course. The 3' and 5' RNA substrates (75 ng each) or AdML pre-mRNA (100 ng) were incubated in splicing reactions (25 μl) (except lanes 7 to 9, which lacked ATP) for 0, 1 or 2 h. RNA was fractionated on an 8% denaturing polyacrylamide gel and detected by phosphorimager analysis. The bands corresponding to splicing intermediates and products are indicated.

METHODS. Plasmids pAdML and pAd5¹³ were linearized with *Bam*HI and *Xho*I, respectively. Plasmids pAd3^{ENH} and pAd3^Δ were constructed by ligating oligonucleotides into the *Bam*HI and *Eco*RI sites of pAd3¹³. Plasmids pAd3^{ENH} and pAd3^Δ were linearized with *Eco*RI. All plasmids were transcribed with T7 RNA polymerase. The *trans* spliced products were identified by RT-PCR and DNA sequencing.



but not between Ad5' and a 3' substrate lacking an enhancer (Ad3^Δ) (lanes 10 to 12). We next constructed Ad3^{ds5'} RNA to determine whether a downstream 5' splice site could substitute for the enhancer to promote *trans* splicing. The intron portion of this 5' splice site is only 7 nucleotides long (Fig. 2a), yet Ad3^{ds5'} *trans* splices to itself when incubated alone under splicing conditions (Fig. 2b, lane 2). The 7-nucleotide 5' intron in Ad3^{ds5'} can also *trans* splice efficiently to the 3' splice site in Ad3^{ENH} (Fig. 2c, lane 2). Thus a 5' intron length of only 7 nucleotides is sufficient for accurate and efficient splicing.

The product generated from *trans* splicing two Ad3^{ds5'} molecules is nearly the same size (193 nucleotides) as that from Ad3^{ds5'}-Ad5' *trans* splicing (189 nucleotides). These two *trans* spliced products migrate as a closely spaced doublet (Fig. 2b, lane 3). However, by incubating ³²P-labelled Ad5' and unlabelled Ad3^{ds5'} under splicing conditions, only the Ad3^{ds5'}-Ad5' *trans* spliced product is detected (Fig. 2d, lane 3). Thus the 5' splice site in Ad3^{ds5'} not only serves as a functional 5' splice site in a splicing reaction, but also serves as an enhancer to promote *trans* splicing to Ad5'. This enabled

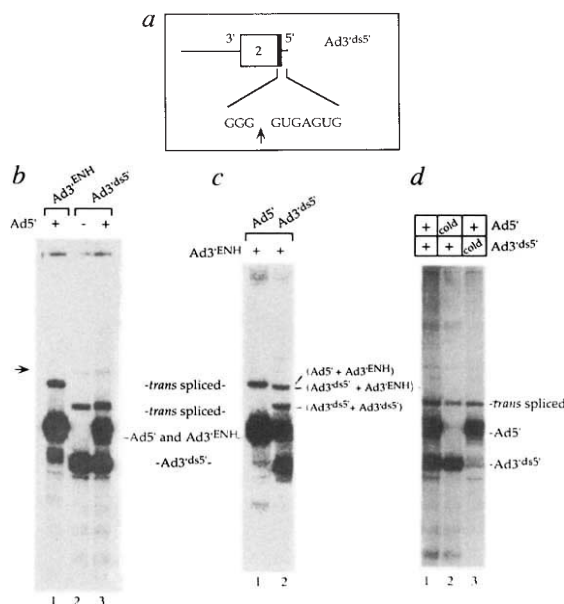


FIG. 2 A 5' splice site can substitute for the exonic enhancer to promote *trans* splicing. **a**, Ad3^{ds5'} structure. Ad3^{ds5'} is the same as Ad3^{ENH} (Fig. 1b) except that a 5' splice-site sequence replaces the enhancer. The arrow indicates the intron-exon border. **b-d**, RNAs (75 ng) were incubated under splicing conditions (25 μl reactions) for 2 h and then fractionated on an 8% denaturing polyacrylamide gel. In **d**, RNAs were either ³²P-labelled (+) or unlabelled (cold). The substrates and *trans* spliced products are indicated. The efficiency of *trans* splicing with the enhancer (see Fig. 1) and the downstream 5' splice site are about the same (7% of the input 5' and 3' substrates are *trans* spliced). The arrow in **b** designates a linear RNA species that, on the basis of its length, is likely to correspond to the *trans* spliced product of Ad3^{ds5'} *trans* spliced to another Ad3^{ds5'}.

METHODS. Plasmid pAd3^{ds5'} was constructed by ligating complementary oligonucleotides into the *Bam*HI and *Eco*RI sites of pAd3¹³. Plasmid DNA was linearized with *Eco*RI and transcribed with T7 RNA polymerase.

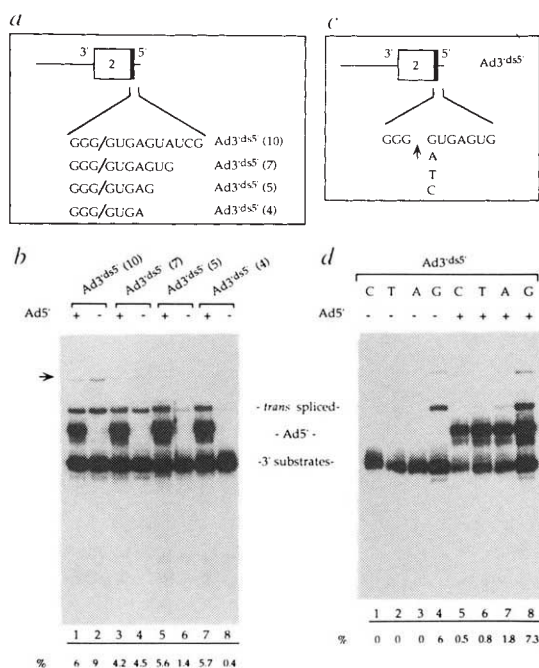


FIG. 3 Effect of 5' intron mutations on enhancer and splicing activities. **a**, Structures of $Ad3^{ds5}$ derivatives containing 5' introns of different length. The sequences and lengths (in nucleotides) of the 5' introns are shown. **b**, $Ad3^{ds5}$ derivatives were incubated in the presence (lanes 1, 3, 5 and 7) or absence (lanes 2, 4, 6 and 8) of $Ad5'$ RNA. The arrow designates the product generated when the *trans* spliced product of $Ad3^{ds5}$ *trans* splices to another $Ad3^{ds5}$ (see Fig. 2b). **c**, Structures of $Ad3^{ds5}$ derivatives containing point mutations in position 1 of the 7-nucleotide intron. **d**, The indicated $Ad3^{ds5}$ substrates were incubated in the absence (lanes 1, 2, 3 and 4) or presence (lanes 5, 6, 7 and 8) of $Ad5'$ under splicing conditions. The efficiencies of each *trans* splicing reaction, determined by phosphorimager analysis, are indicated (% spliced/unspliced).

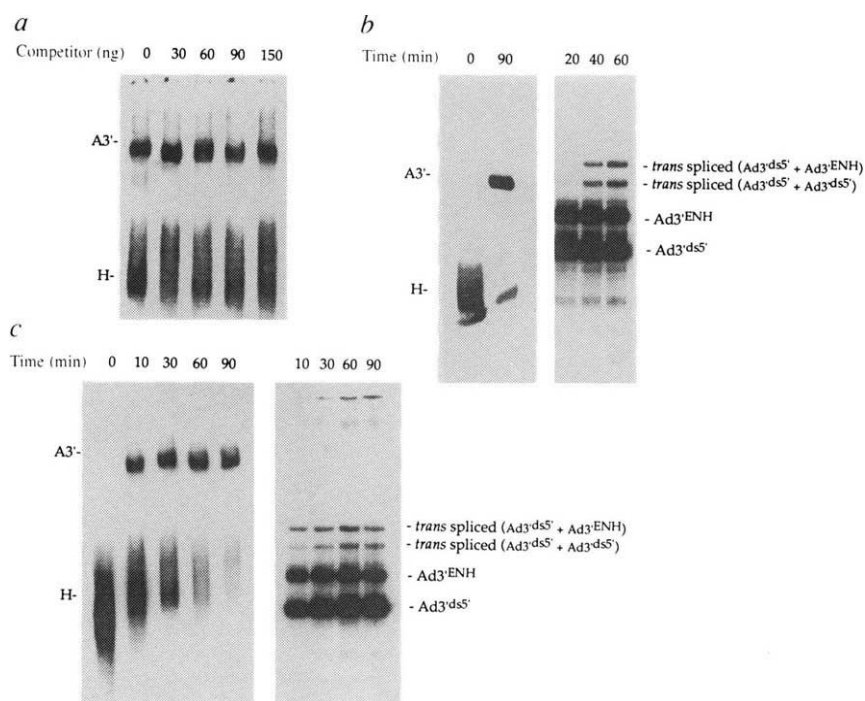
METHODS. Plasmid $pAd3^{ds5}$ derivatives containing the 5' introns of different length were constructed by cloning oligonucleotides into $pAd3'$. The correct lengths of the 5' introns were generated by truncation of the plasmids with appropriate restriction enzymes followed by transcription with T7 RNA polymerase. The $pAd3^{ds5}$ containing the intron position 1 point mutations were constructed by inserting the appropriate oligonucleotides containing either A, C, or U into the *Bam*HI and *Eco*RI sites of $pAd3'$.

us to determine the effect of 5' intron length on these two activities. Derivatives of $Ad3^{ds5}$ that contain 5' introns of different length (Fig. 3a) were incubated alone under splicing conditions. This revealed that 5' intron lengths of 4 or 5 nucleotides have significantly less splicing activity than intron lengths of 7 or 10 nucleotides (Fig. 3b, lanes 2, 4, 6 and 8). In contrast, incubation of the derivatives with $Ad5'$ revealed that the introns of 4 or 5 nucleotides can still function efficiently as enhancers to promote *trans* splicing (Fig. 3b, lanes 5 and 7). Thus these intron truncations uncouple the enhancer activity of the 5' intron from its splicing activity. In *cis* spliced pre-mRNAs, the enhancer activity of downstream 5' splice sites requires binding of U1 small nuclear ribonucleoprotein particles

(snRNPs)^{1,4}. Our data are consistent with this as even the 4-nucleotide intron has the potential to form 6 out of 8 contiguous base pairs with U1 snRNA.

U1 is thought to be replaced by U6 snRNP at the 5' splice site before catalytic step I of the splicing reaction⁵⁻⁷. Our data indicate that any essential role for U6 snRNP at the 5' splice site is restricted to the first 4 nucleotides of the intron. Consistent with this, crosslinking studies have detected an interaction between U6 snRNA and the first 4 nucleotides of the intron before catalytic step I in yeast (C. H. Kim and J. Abelson, personal communication), whereas a U6 snRNA interaction with intron position 2 is detected before catalytic step II in mammals⁵. Finally, our data support the idea that a U6 snRNA

FIG. 4 Selection of the *trans* 5' splice site can occur after assembly of the pre-spliceosomal complex A on the 3' substrate. **a**, $Ad3^{ENH}$ (180 ng) was incubated under splicing conditions (60 μ l reaction) for 90' to form A3' complex. The indicated amounts of cold $Ad3^{ENH}$ (competitor) were then added to 10- μ l portions of this A3' reaction together with splicing extract, and incubation was continued for 60 min (final reaction volume was 15 μ l). Samples were fractionated on a native gel. **b**, $Ad3^{ENH}$ (150 ng) was incubated for 0 min or 90 min under splicing conditions (50- μ l reactions), and 10- μ l portions were fractionated on a native gel (left). $Ad3^{ds5}$ (30 ng) was added to 10- μ l portions of the 90-min time point (left) together with splicing extract (15- μ l final reaction volume), and incubation was continued for 60 min (final reaction volume was 15 μ l). RNA was fractionated on an 8% denaturing polyacrylamide gel. **c**, $Ad3^{ENH}$ (360 ng) was incubated under splicing conditions (120- μ l reaction) for the times indicated, and portions were fractionated on a native gel (left). $Ad3^{ds5}$ (30 ng) was added to portions (10 μ l) of each sample (left) together with splicing extract, and incubation was continued for 60 min (final reaction volume was 15 μ l). RNA was fractionated on an 8% denaturing polyacrylamide gel. The A3' complex and the heterogeneous nuclear ribonucleoprotein complex (H) are indicated in the native gels, and the substrates and *trans* spliced products are designated for the polyacrylamide gels.



duplex formed with intron positions 4 to 6 contributes to splicing efficiency, but is not essential for the reaction⁶⁻⁹.

Mutation of the invariant G at position 1 of the 5' intron to A, C, or U severely decreases its enhancer activity (Fig. 3c, d, lanes 5 to 8), probably because the U1 snRNA-5' splice site duplex is disrupted in the middle. The splicing activity of all three 5' intron position 1 mutants is abolished (Fig. 3c, d, lanes 1 to 4). This loss of splicing activity is not due solely to the decrease in enhancer activity of the 5' intron, as the position 1 mutants also do not *trans* splice to Ad3^{ENH} (data not shown).

It has been reported^{10,11} that *trans* splicing in mammals can only occur if the 5' and 3' substrate RNAs form base pairs by means of a segment of complementarity in their introns. However, our data contradict this. Not only does the 5' substrate containing the 7-nucleotide intron have no complementarity to the intron in the 3' substrates, but it is also highly unlikely that such a short 5' intron could function in splicing while base-paired to the 3' intron. Another study¹² reports *trans* splicing without apparent complementarity between the 5' and 3' introns. The 3' substrate used¹² fortuitously contained a 5' splice site on the downstream end of exon 2. This probably explains their detection of *trans* splicing in the absence of complementarity between the introns.

We next investigated whether pairing of the *trans* 5' and 3' splice sites must occur during assembly of the earliest pre-spliceosomal complex E^{13,15}, or whether it can occur after the second pre-spliceosomal complex A assembles on the 3' substrate. As expected¹⁶, the A3' complex assembles efficiently on AD3^{ENH} (Fig. 4a, lane 0) and is stable to the addition of excess cold competitor and continued incubation (Fig. 4a, lanes 30–150). To determine whether *trans* splicing can occur after irreversible assembly of the A3' complex, we formed this complex on Ad3^{ENH} (Fig. 4b, left, lane 90), added naked Ad3^{ds5'} and continued incubation under splicing conditions for the times indicated (Fig. 4b, right). The data show that Ad3^{ds5'} *trans* splices as efficiently to Ad3^{ENH} pre-assembled into the A3' complex as to naked Ad3^{ds5'}. No kinetic advantage of pre-assembling the A3' complex is observed, probably because there is a rate-limiting step for *trans* splicing which follows A3' complex assembly. When Ad3^{ENH} is incubated for different times to form the A3' complex (Fig. 4c, left, lanes 10 to 90), followed by addition of naked Ad3^{ds5'} RNA, *trans* splicing of Ad3^{ENH} to Ad3^{ds5'} occurs with the same efficiency at all time points (Fig. 4c, right). Together these data demonstrate that pairing of the *trans* 5' and 3' splice sites can occur after A3' complex assembly.

In yeast pre-mRNAs¹⁴, and in mammalian pre-mRNAs containing short introns^{13,15}, the 5' and 3' splice sites are paired in the E complex. However, metazoan pre-mRNAs typically contain introns as large as 10⁴ or 10⁵ nucleotides, raising the question of how splice sites come together over such long distances. In this study we have shown that mammalian 5' and 3' splice sites located on separate synthetic pre-mRNA substrates can be efficiently *trans* spliced if the 3' *trans*-splicing substrate contains specific elements (a downstream 5' splice site^{1,4} or an exonic enhancer^{2,3}) implicated in splicing pre-mRNAs containing long introns¹ (see Fig. 1a). Moreover, selection of the *trans* 5' splice site can occur after the A complex has assembled on the *trans* 3' substrate. Thus these observations suggest a model in which 5' and 3' splice-site pairing in metazoan pre-mRNAs containing very long introns may occur in two distinct steps. □

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ACKNOWLEDGEMENTS. We thank R. Feld for technical assistance, and members of our laboratory and V. Andres for discussions and comments on the manuscript. M.D.C. is supported by the Spanish Ministry of Education and Science. This work was supported by a Lucille P. Markey Scholar award and a National Institutes of Health grant.

Direct NMR evidence for an intermediate preceding the rate-limiting step in the unfolding of ribonuclease A

Thomas Kiefhaber*, Alexander M. Labhardt† & Robert L. Baldwin‡

* Abteilung für Biophysikalische Chemie, Biozentrum der Universität, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

† Pharma Research New Technologies, Hoffmann-LaRoche Co., Basel, Switzerland

‡ Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305-5307, USA

It is commonly believed that there are no detectable intermediates in the kinetic unfolding reactions of small proteins¹⁻⁶. If such intermediates could be found, they would give important information about the nature of the transition state for unfolding, which is thought to occur close to the native state. We report here that one-dimensional proton magnetic resonance spectra recorded during the unfolding of ribonuclease A provide direct evidence for at least one unfolding intermediate in which side chains are free to rotate. This intermediate appears to be a 'dry molten globule' of the kind hypothesized by Shakhnovich and Finkelstein⁷.

The basic reason for concluding that unfolding intermediates probably do not exist is that unfolding monitored by optical probes follows a single exponential curve (if there are no complicating factors such as proline isomerization after unfolding), and the same unfolding curve is produced by monitoring probes either of secondary structure or of tertiary structure⁸. This is illustrated in Fig. 1 for the unfolding reaction of ribonuclease A (RNase A) studied here: circular dichroism (CD) follows the same unfolding curve when observed either at 222 nm, which monitors α -helix unfolding, or at 275 nm, which monitors the release of buried tyrosine side chains from fixed positions in the tertiary structure. This unfolding behaviour should be contrasted with the behaviour of these same two probes in the refolding reactions of most small proteins when they give very different kinetic curves⁹.

Nevertheless, the chemical shifts of resolved proton resonances in NMR spectra provide much more sensitive probes for changes in conformation and might detect unfolding intermediates. In the unfolding conditions chosen here, the unfolding process is slow enough that one-dimensional (1D) spectra can be recorded. When the CD-monitored unfolding curve shown in Fig. 1 is fitted to a single exponential, the relaxation time is $\tau = 320$ s. A 1D ¹H-NMR spectrum can be recorded in 42 s and the first spectrum can be started about one minute after the beginning of unfolding when the process is initiated by adding a small amount of solution containing native protein to one that contains concentrated guanidinium chloride (GdmCl). Figure 2b shows the spectrum recorded 109 s (midpoint) after the start of unfolding: at this time, 70% of the protein is still folded accord-

Received 15 November 1994; accepted 19 April 1995.

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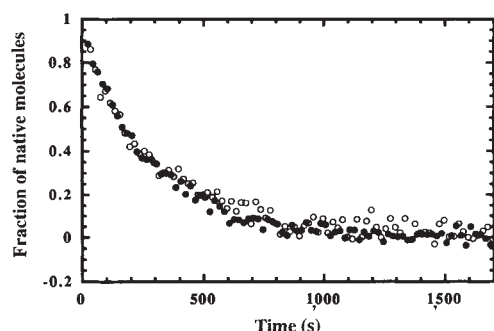


FIG. 1 Unfolding of RNase A at pH 8.0, 10 °C, 4.5 M GdmCl, 50 mM Tris Cl, D₂O, monitored by CD at two wavelengths: 222 nm (●) and 275 nm (○). The protein concentration was 1 mg ml⁻¹; identical results at 275 nm were obtained with protein concentrations of 5 mg ml⁻¹ and 10 mg ml⁻¹. Both probes give the same unfolding curve within experimental error. Fitting to a single exponential (not shown) gives the relaxation time $\tau = 320$ s.

ing to the CD results in Fig. 1. For reference, Fig. 2a shows the equilibrium spectrum of the unfolded protein in the same conditions and Fig. 2c shows the spectrum of the native protein in 3.0 M GdmCl, at the edge of the unfolding transition zone. (The transition zone extends from 3.0–4.0 M GdmCl, data not shown.) The spectrum in Fig. 2b closely resembles the unfolded spectrum, although native resonance lines can still be observed with low intensities. An equilibrium spectrum taken at 3.5 M GdmCl, near the midpoint of the unfolding transition, resembles a weighted mixture of the spectra of the native and unfolded forms.

A C^γH₃ resonance line of Val 63 and C^δH₂ resonance line of Pro 117 are resolved in the native spectrum near 0.4 p.p.m. and 0.1 p.p.m., respectively. The positions of these lines (Fig. 3a) and also their intensities in native RNase A are independent of GdmCl concentration and they are observed in the first 1D spectrum recorded during unfolding (Figs 2b and 4). The intensity of the Val 63 line after 109 s of unfolding in 4.5 M GdmCl is, however, only 26% of that in the native protein, although 70% of the molecules should be native according to CD measurements (Fig. 1). In an unfolding experiment at 4.3 M GdmCl, the number of scans per spectrum was reduced from 32 to 16 in order to record a spectrum in 21 s and get better time resolution. The midpoint of the first spectrum (Fig. 4) was 82 s after the start of unfolding, when 85% native molecules should be present according to CD data but only 30% native molecules are present according to the intensity of the Val 63 resonance. Similar results were obtained for the Pro 117 resonance, but the data are less accurate because of the lower intensity of the Pro 117 line. In Fig. 4, the aromatic region and the C^αH region of the spectrum are partly obscured by a residual water peak at 4.8 p.p.m. and by a Gdm⁺ peak at 6.8 p.p.m. caused by exchange with the residual water.

The time course of disappearance of the Val 63 resonance is shown in Fig. 3b, both at 4.5 M and 5.8 M GdmCl. The relative amount of native protein extrapolated back to zero time of unfolding is 0.35 at 4.5 M and 0.12 at 5.8 M. Except for this initial drop, the kinetics of disappearance of the lines of the native protein agree with the kinetics measured by CD. The results can be explained by a simple kinetic model for unfolding



in which an intermediate I is formed rapidly from the native state, but shows slow exchange with N (>ms) on the NMR time scale. The methyl and methylene resonances of I resemble those of U. More than one intermediate may be present. A single intermediate is given in equation 1 because this is the simplest

model that fits the present data. The 'global' unfolding reaction occurs from the $N \leftrightarrow I$ equilibrium mixture and leads to completely unfolded protein, U. If this model is correct, then the rapid decrease in the amount of N at the start of unfolding should be caused by N becoming increasingly less stable than I at increasing concentrations of GdmCl. Probably GdmCl interacts more strongly with I than with N because more sites for interaction are exposed in I than N. Preliminary data show that I is not populated at equilibrium: the results will be published elsewhere.

These results are quite surprising in view of the widespread belief that there are no observable intermediates in protein unfolding. It is important to consider if there can be an artefact. The following points are relevant. First, the NMR unfolding experiment has been repeated, with the same results, and the same pattern of results has been obtained at 4.3, 4.5 and 5.8 M GdmCl. Second, unfolding has been monitored by CD (Fig. 1) and by NMR (Fig. 2) in identical solvent conditions. Third, unfolding has been monitored by near-ultraviolet CD at a protein concentration similar to that used in the NMR experiment as well as at lower protein concentrations, and the unfolding kinetics are independent of protein concentration (data not shown). Fourth, the technique used to record the NMR spectrum (the number of scans per spectrum and the time interval between scans) is the same in the kinetic unfolding experiment (Fig. 2b) as in the spectra taken at equilibrium (Fig. 2a, c). Fifth, the intensities of the V 63 and P 117 resonance lines are nearly independent of GdmCl concentration between 0.5 M and 3 M

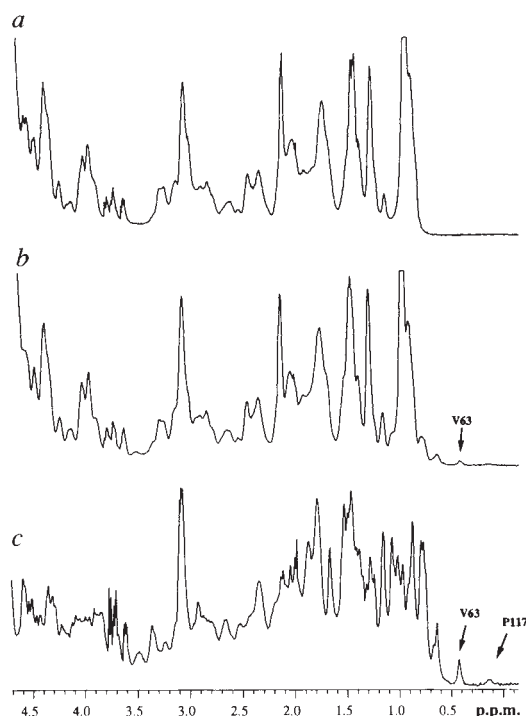


FIG. 2 Unfolding intermediate shown by 1D ¹H-NMR. A spectrum started 88 s after the beginning of unfolding is shown in b; recording the spectrum took 42 s, and the time at the midpoint of spectral collection was 109 s. Conditions: 4.5 M GdmCl, pH* 8.0 (not corrected for the isotope effect), 10 °C, D₂O, 50 mM Tris Cl, 10 mg ml⁻¹ protein. For comparison, a spectrum taken at 3.0 M GdmCl, which shows about 95% native protein, is given in c, and a spectrum of the unfolded protein at equilibrium in 4.5 M GdmCl is given in a. The methylene region of the spectrum is shown. The unfolding intermediate has a spectrum closely resembling that of the unfolded protein. Spectra were recorded on a Bruker AMX II 600 MHz spectrometer; 32 scans were averaged to give each spectrum. The assignments of the V 63 and P 117 resonances were given^{16,17}.

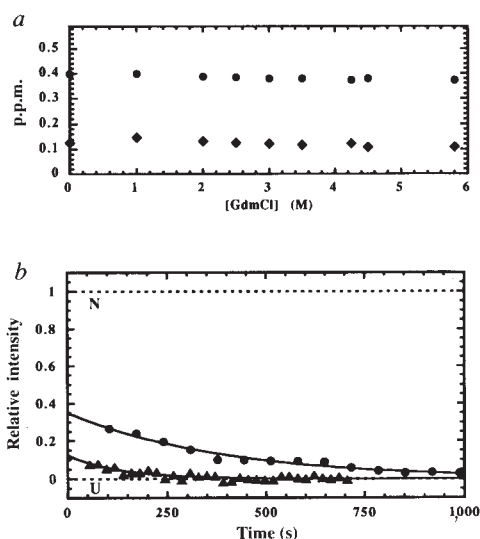


FIG. 3 a, Dependence on GdmCl concentration of the chemical shifts of a CH₃ resonance of Val 63 (○) and of the CH₂ resonance of Pro 117 (◆), at pH* 8.0, 10 °C, D₂O. b, Kinetics of disappearance during unfolding of a native CH₃ resonance of Val 63 at 0.4 p.p.m.: (●) 4.5 M GdmCl, (▲) 5.8 M GdmCl. Consecutive spectra consisting of 32 scans (4.5 M) or 16 scans (5.8 M) were recorded. The reference spectra used to normalize the peak intensities were also accumulated 32 times or 16 times respectively. Peak integration was made using an automated baseline fit/integration procedure using the program NDEE and, additionally, by manual integration using the program UXNMR. Small shifts in line positions occur at early times. When these are taken into account, both procedures give essentially identical results.

(data not shown) so that the rapid decrease in their intensities at the start of unfolding is not a direct effect of GdmCl on native RNase A. Finally, similar results have been obtained with a different protein (hen egg-white lysozyme, D. V. Laurents, and R.L.B., unpublished results) in conditions where the kinetics of unfolding are considerably slower than those shown here for RNase A.

The unfolding intermediate is detected by the loss of chemical shift dispersion of side-chain resonances. The distinct chemical shifts of protons in the native spectra arise from unique magnetic environments in the close-packed native structure. There are small contact shifts, especially from heteroatoms, and larger ring current shifts from aromatic residues that need not be in contact¹⁰. The chemical shift dispersion is lost in the unfolded protein because contact shifts are lost when side chains are free to rotate and ring current shifts are reduced as the protein expands and are averaged when the side chains are free to rotate. Equilibrium molten globule intermediates also show drastically

reduced chemical shift dispersion and their spectra resemble the spectra of unfolded proteins¹¹.

There are two basic differences between the unfolding intermediate detected here and most equilibrium ('wet') molten globule intermediates. First, retention of the near-UV CD spectrum by the unfolding intermediate (see Fig. 1) shows that the buried tyrosine side chains of RNase A remain buried in the unfolding intermediate. Loss of the near-UV CD spectrum as the aromatic side chains become partly or fully exposed to solvent is standard behaviour shown by most equilibrium molten globule intermediates¹¹. (A molten globule form of equine lysozyme is, however, reported to retain its near-UV CD spectrum¹².) Second, NMR hydrogen exchange (²H-¹H) experiments, monitoring exchange of individual peptide NH resonances during unfolding at pH 8.0 and pH 9.0, 10 °C, 4.5 M GdmCl, show that protons in the unfolding intermediate are resistant to exchange with solvent and exchange does not occur until the rate-limiting step in unfolding is reached¹³, whereas experimental results show that those equilibrium molten globule intermediates that have been studied are only weakly protected against exchange^{14,15} and would show rapid hydrogen exchange in these conditions. These two differences indicate that the present unfolding intermediate is a 'dry' molten globule: water has not yet penetrated the hydrophobic cores of RNase A, although most side chains have become free to rotate. The loss of stability that occurs when water penetrates the cores is responsible for the rapid hydrogen exchange seen in equilibrium molten globule intermediates. If the unfolding intermediate reported here is a solvated molten globule, it should show rapid hydrogen exchange and this is not observed.

Two hypotheses on the nature of the rate-limiting step have been widely discussed. The first is the 'dry molten globule hypothesis'⁷. The protein expands under the influence of thermal fluctuations. At a critical degree of expansion, side chains become free to rotate although water cannot yet enter the hydrophobic cores. This has been postulated to be the rate-limiting step in unfolding⁷. Our results indicate that a dry molten globule is formed during unfolding in GdmCl, but that it is a populated intermediate which precedes the rate-limiting step. The second hypothesis is that the rate-limiting step occurs when water enters the hydrophobic cores. Then the protein becomes unstable in denaturing conditions and overall unfolding occurs rapidly. Our results are consistent with this hypothesis. We show elsewhere that, when the kinetics of unfolding are monitored by hydrogen exchange, the entire network of peptide hydrogen bonds breaks in a single kinetic event¹³ and exchange seems to be rate limited by the same unfolding step that is monitored by CD, indicating that overall unfolding takes place in this step. These results suggest that breaking the hydrogen bond network, rather than the loss of side chain interactions, is the rate-limiting step in protein unfolding. □

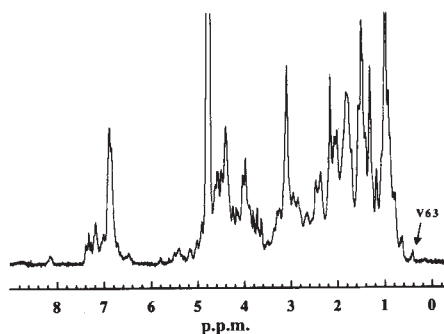


FIG. 4 Spectrum taken 82 s (midpoint) after the beginning of unfolding in 4.3 M GdmCl, pH* 8.0, 10 °C; 16 scans were accumulated and averaged. There is a residual water peak at 4.8 p.p.m. and a residual (¹H) Gdm⁺ peak at 6.8 p.p.m.

Received 14 February; accepted 4 April 1995.

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ACKNOWLEDGEMENTS. We thank P. Bayer and D. Laurents for help and discussion. Our work was supported by an NIH grant and by a grant from the Schweizerische Nationalfonds.

Small products of polymerization

Although the subject is somewhat fragmented, product review comes in with a full line-up of products — a DNA sequencer, sequence analysis software, modified nucleic acids and amino acids and an electroporator.

INTELLIGENETICS offers a brochure describing the GeneWorks **sequence-centred analysis software** (*Reader Service No. 100*). This eight page, colour brochure shows how this program can be used for DNA and protein sequence analysis for the Macintosh. GeneWork's multiple-view linkage is designed to allow the user to perform many analyses simultaneously. There are illustrations with screens and graphics that demonstrate the program's capabilities in database searching, multiple sequence alignment, dot matrix similarity, circular mapping, PCR priming, as well as complete protein analysis functionality.

Perkin-Elmer has introduced the Phosphalink **non-nucleosidic phosphoramidite** that directly incorporates a phosphate at either the 3' or 5' end, or both, of chemically synthesized oligonucleotides (*Reader Service No. 101*). It presents an efficient alternative to labour-intensive enzymatic methods, because phosphorylation occurs during synthesis, and it requires no more time than any other base addition. This is designed to eliminate enzymatic phosphorylation that can add hours of post-synthetic processing. This phosphoramidite is packaged for use on all Perkin-Elmer nucleic acid synthesizers. Only 5' phosphorylated oligonucleotides will ligate in the presence of T4 DNA ligase. This product extends the flexibility and control obtainable with synthetic DNA to all ligation experiments, including LCA, OLA, gene construction, cloning and sequencing cDNA to the 5' end.

The Zero background cloning kit from Invitrogen utilizes the **cloning vector pZER0-1**, which allows positive selection of inserts by disrupting a highly lethal gene (*Reader Service No. 102*). When an insert is cloned into this vector, only recombinants are permitted to grow. The kit is designed to offer high cloning efficiencies and low background without vector dephosphorylation or blue/white colour screening with X-gal.



pZER0-1 vector.

Wallac's Delfia Eu-oligolabelling kit allows **europium labelling of amino-mod-**



Wallac's Delfia Eu-oligolabelling kit.

ified oligonucleotides for use in hybridization assays (*Reader Service No. 103*). The kit is intended to help researchers who have already established a probe, and who need to screen a large number of samples either as a batch or over a longer period of time. Typical applications include the detection of viruses or genetic diseases, HLA-typing and anti-biotic resistance. It allows labelling of about 100 µg of 20-base oligonucleotide — the specific activity, depending on the number of amino groups, will vary from between 10 and 30 Eu³⁺ per oligonucleotide. The use of microtitre plates as a solid support means that hundreds of samples can be handled simultaneously. The product consists of 5 mg of labelling reagent, 0.5 ml of Eu-standard, one bottle each of enhancement solution, assay buffer and wash concentrate, and a microtitre plate.

■ Oligonucleotide synthesis

The Oligo 1000 from Beckman is engineered to be an affordable and cost-efficient **DNA synthesizer** capable of synthesizing stat oligonucleotides or special chemistries like RNA and on-line labelling (*Reader Service No. 104*). Also available is the Oligo 1000M, designed for individuals with high-volume requirements or groups of individuals. This synthesis technology is said to allow the user to produce high-quality oligonucleotides in less than one hour with two-minute cycle times. It is designed to need no dedicated operator, to automate reagent monitoring, to predict usage, to track age, to detect empty bottles and to prevent waste spillage. A choice of phosphoramidite packaging includes ready-

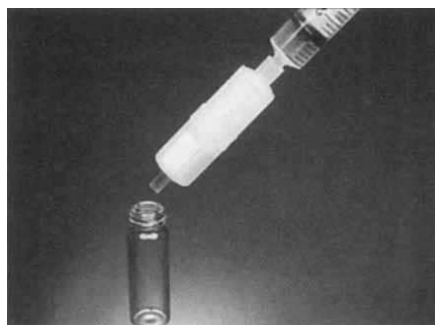
to-mix, moisture-proof Binary Paks or economical Mono Paks. The company states that the system uses less of the expensive reagents and uses a 30-nmol scale for PCR.

New from BioGenex Laboratories — **2'-O-methyl RNA Phosphoramidites** for the efficient **synthesis of RNA oligonucleotides** with improved characteristics for antisense research and other applications (*Reader Service No. 105*). RNA oligonucleotides containing the 2'-O-methyl modification are said to have increased resistance to nucleases, enhanced cell membrane permeability and a higher affinity to the target sequence than comparable unmodified RNA or DNA oligos. It is these properties that make 2'-O-methyl RNA analogues suitable for antisense, ribozyme and triplex research, as well as for RNA enrichment and other hybridization applications. The new reagents are packaged for convenient attachment to the ports on most DNA synthesizers. They are supplied as a dry powder in a form ready for dissolution with anhydrous acetonitrile. Both 0.1-gram and 0.25-gram sizes are available.

■ Purification

Boehringer Mannheim has developed antidigoxigenin magnetic particles for the **separation of digoxigenin-labelled biomolecules** (*Reader Service No. 106*). Within one minute of application, the antidigoxigenin magnetic particles and their attached labelled molecules can be separated out of the sample because of the antibodies' high affinities for the digoxigenin label. With this particle separation technology, molecular biologists can separate out oligonucleotide, DNA fragments, proteins, peptides and glycoconjugates.

Cambio offers Poly-Pak cartridges (from Glen Research) which provide a means of **purifying synthetic oligonucleotides**, including amino-modified, oligo-phosphorothioates, biotinylated, dye-



Cambio's Poly-Pak cartridges.

PRODUCT REVIEW

labelled and thio-labelled (*Reader Service No. 107*). The method is based on hydrophilic interaction chromatography and is designed to give high product recovery in low eluate volumes — typically 50 absorbance units in 0.5 ml of solvent on a synthetic scale of 1 μ mol, says Cambio. In addition, the method is said to improve sequencing by eliminating interference from failed oligonucleotide sequences and removes deprotecting ammonia without damage from a vacuum pump.

■ Peptides

AnaSpec, which offers **custom peptide synthesis**, reagents and resins, has now added mass spectrometry to its list of services (*Reader Service No. 108*). The company is using MALDI time-of-flight mass spectrometry to analyse peptides, proteins, glycoproteins, lipids, oligosaccharides, oligonucleotides, polymers, and chemical and proteolytic digests. AnaSpec says its mass spectrometer measures down to femtomole quantities with resolution from 50 to 300 daltons, depending on the sample mass. In addition, masses from between 400 and 150,000 daltons can be measured, with stated accuracies of 0.1 to 0.001 per cent. The turn-around time for samples is 24 hours.

Synthesize up to 96 peptides on a cellulose sheet with the SPOTs **epitope analysis system** from Genosys Biotechnologies (*Reader Service No. 109*). The peptides bound to the sheet can be probed with antibody. Stated applications include epitope scanning and analogue and boundary analysis. Each 8 $\times$ 12 cm sheet contains 96 pre-activated 'spots'. Individual peptides are synthesized at each of these pre-activated spots. Fmoc amino acid active ester chemistry ensures high synthetic coupling efficiency. Moreover, computer software is supplied with the system that enables the user to follow the synthesis schedule. Once a set of peptides is synthesized, each spot can be probed with antibody just like a dot blot. Use of a secondary antibody enzyme conjugate with an indicator dye then reveals those spots that contain the peptide representing the epitope detected by the antibody. The sheet can be regenerated and re-probed several times. The SPOTs kit is supplied with all the reagents necessary to begin peptide assembly; replacement components are offered separately, in addition to an assortment of β -galactosidase antibody conjugates.

Oxford Asymmetry offers a **range of beta amino acids and β -amino- α -**

hydroxy acids (*Reader Service No. 110*). The company will supply custom amino acids on request — both enantiomers with Fmoc or Boc protection in quantities from grams to kilograms. These products are said to be of high chemical, enantiomeric and diastereomeric purity.

Bachem now has available the **amyloid β -proteins** 1–40, 1–42 and 1–43, as well as many other fragments (*Reader Service No. 111*). Specific fragments of the amyloid beta-protein precursor molecule are also supplied. These protein fragments may prove useful for studying the effects of malignant plaques. These peptides are derived from the amyloid precursor protein, which is broken down by different pathways into peptides of different length.

■ Labelling and quantitation

A new line of **fluorescently labelled primers** is now available from PanVera (*Reader Service No. 112*). According to the company, these oligonucleotides are labelled to a very high specific activity (typically 60–80 per cent) and are complementary to widely used plasmid primer sites making them suitable for synthesis of fluorescent DNA probes from most cloning vectors. The sequences can be labelled at one or

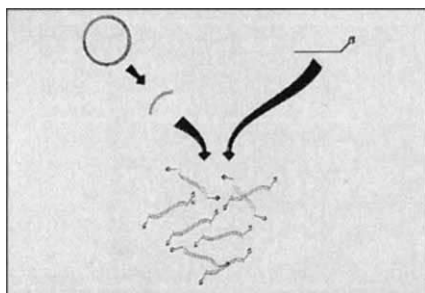
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Brian Ward, Ph.D.

- Presently working on DNA sequence recognition methods.
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- Solved dideoxy sequencing stalling by temperature ramping the termination reactions.
- Has worked in the Molecular Biology Department of Sigma for eight years.
- Received his Ph.D. from Michigan State University.



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Pan Vera's fluorolabelled primers.

both ends, depending on the application. PanVera says that these primers are useful for detection using fluorescence polarization techniques because of the short spacer arm that is incorporated in the phosphoramidite and the high specific activity. Fluorescein-labelled amplified products can be used for fluorescence *in situ* hybridization or in direct detection by simple fluorescence. Suitable for use in various enzymatic labelling methods, including PCR, the available oligos include T7, SP6, M13 Forward, M13 Reverse and Oligo dT.

New from Molecular Probes is OliGreen, an **oligonucleotide quantitation reagent** for single-stranded DNA (Reader Service No. 113). It is said to

detect 100 pg ml⁻¹ ssDNA or oligomers using a standard spectrofluorometer that is fitted with fluorescein-compatible filters. Fluorescence microtitre plate readers can also be used with this reagent. According to the company, the assay is linear over four orders of magnitude, from 100 pg ml⁻¹ to 1 µg ml⁻¹, without adjusting the dye concentration. Moreover, the assay is said to be relatively insensitive to the effects of detergents, salts and other compounds that are commonly used in enzymatic reactions. OliGreen is supplied with a protocol and enough reagent for 200 assays (using a standard fluorometer cuvette).

Also from BioGenex Laboratories — Biotin CED Phosphoramidite and Fluorescein CED Phosphoramidite for the **synthesis of oligonucleotides labelled with biotin and fluorescein** (Reader Service No. 114). These two reagents can be used like conventional DNA phosphoramidite monomers: no modification of the standard synthesis and deprotection protocols is required. According to the company, coupling efficiencies of greater than 98 per cent can be achieved. The new reagents can be used to synthesize oligos with biotin or

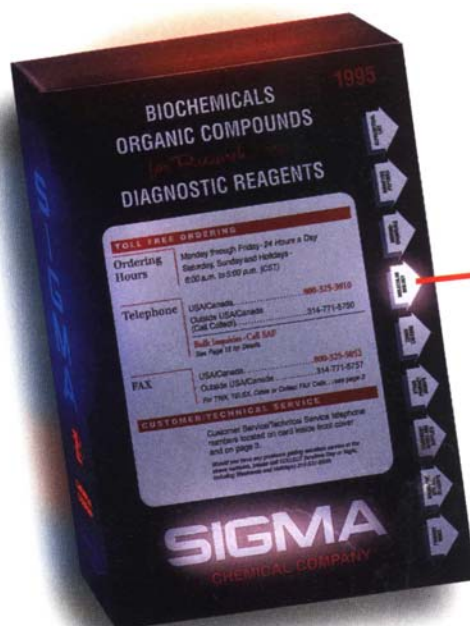
fluorescein labels at the 5' end, internal positions, or both. Stated uses include DNA probe synthesis, non-radioactive analysis, and primer extension protocols such as PCR and DNA sequencing. The reagents, which are packaged for attachment to the ports of most DNA synthesizers, are available in 0.05-mmol, 0.1-mmol and 0.25-g sizes.

The DNA Quant 200 fluorometer from Hoefer Scientific is designed to provide a rapid, accurate and cost-effective means of **quantitating small volumes of DNA** (Reader Service No. 115). The instrument uses the site-specific binding properties of the Hoechst 33258 dye for the assay. Sensitivity is said to be 200–300 times greater than ultraviolet spectroscopy, without problems of interference of RNA, proteins or nucleotides. It should be useful in PCR amplification, cell lysates, plasmid/phage separations, RFLP analysis, sequencing and sub-cloning applications. Capillary sample



DNA Quant 200.

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"Even though the products of this group have always been right here."

Brian is presently concentrating his efforts on DNA sequence recognition methodologies. In addition, he is also an important part of a team working with nucleic acid isolation, recombinant protein expression, and transcription/translation technologies. You can find the products of Brian's work, as well as his co-workers', in the Molecular Biology section of the Sigma Catalog. And just like all the Sigma products you know and trust, your satisfaction is guaranteed.

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cell accessories are available to accommodate sample volumes down to 3 µl, with as little as 1 ng of total DNA.

R&D Systems can provide **oligo-nucleotides** of high-quality DNA with a wide range of modifications (*Reader Service No. 116*). The company's services include a choice of synthesis scales — 0.04, 0.2, 1.0, 2.5, 10.0 and 25 µm; purification methods, PAGE, HPLC, RP cartridges and FPLC; and DNA modifications. A variety of haptens allows the user to reduce or eliminate radioactivity in the laboratory; these include digoxigenin, biotin, fluorescein rhodamine, Texas red, acridine, ABI dye and DNP. Oligo-nucleotides linked to alkaline phosphatase, horseradish peroxidase and bovine serum albumin are also available. The company can also provide a variety of linker molecules so that researchers can attach haptens in their own laboratory. There are two nuclease-resistant structures offered, as well as cholesterol.

■ Laboratory equipment

The Hermle Z233 M and the Z233 MK **centrifuges** have been designed with microprocessor control, a brushless inductive drive and four available rotors (*Reader Service No. 117*). The maximum capacity of these models is 44 × 2.0 ml with a maximum speed of 13,500 r.p.m. for the Z233



The Z233 M and Z233MK centrifuges.

M non-refrigerated unit and 14,000 r.p.m. for the Z233 MK refrigerated model. The highest speed can be reached in as little as 10 s and the display may be changed from 'speed' to 'g value' with the touch of a button.

The new Eppendorf **electroporator** 2510 is designed to transform bacteria quickly, efficiently and inexpensively (*Reader Service No. 118*). The unit has a footprint of only 23 × 27 cm. There are no additional components required and there is no need for potentially dangerous high-voltage leads. The 2510 electroporator has a voltage range of 200–2,500 V and an exponential decay pulse with a fixed time constant of 5 milliseconds. A one-button capacitor is automatically



Eppendorf's 2510 electroporator.

charged and the pulse is delivered to the sample chamber within eight seconds (at maximum voltage). After the pulse is delivered, the actual voltage and time constant are displayed on the digital readout and are also sent to an optional printer to provide a hard copy of experimental parameters.

Pick up a copy of Genemed's **custom synthesis catalogue** (*Reader Service No. 119*). This catalogue is intended to provide researchers with an easy-to-use guide that specifies which service is best suited to a particular application — whether for PCR scale or research, standard or large scale. The catalogue covers methods for purification, modification, labelling and analysis. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

Erratum

In column one of Checovich *et al.* (*Nature* 375, 254–256; 1995), "Polarization value μ rotational relaxation time is proportional to $3\eta V/RT$ " should have read, "Polarization value $\proq$ relaxation time = $3\eta V/RT$ ".

In column two on page 255 "(p,[DNA], K_d)" should have been omitted.

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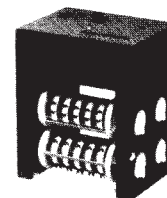
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